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**DEVELOPMENT OF A NOVEL NANOTECHNOLOGY
BASED ARTIFICIAL ANTIGEN PRESENTING CELL
SYSTEM FOR ADOPTIVE AND ACTIVE
IMMUNOTHERAPY**

D R. H U G O D E L A P E Ñ A, M D.

**A Thesis Submitted To The University Of London For The Degree
Of Doctor of Philosophy**

**Anthony Nolan Research Institute
Royal Free And University College Medical School
University Of London, United Kingdom**

2007

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indicated in the thesis

DEDICATION

The PhD has been a test of endurance, strength and perseverance. Thank you mum and dad for having given me exactly that example all my life. You always said I had a big star looking after me. You were right; it was you all the time. Thank you so much. I love you. I would not be half of the person I am without you.

To God, my best friend. All I am I owe to you. Thank you.

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PHD ABSTRACT

T cells are one of the most pivotal cell types in the human adaptive immune system. They have the capacity to eradicate primary, metastatic, relapsed tumours and can ameliorate otherwise fatal viral infections. Not surprisingly therefore, the activation and expansion of T cells has become one of the main focuses for immunotherapy and immune gene therapy. However one of the problems of T cell mediated immunotherapy in terms of delivering significant clinical impact to patients, is the expansion of high numbers of functional antigen specific effector T cells. The current approaches for expanding T cells have a number of drawbacks in terms of timing, reproducibility and reliability. Many if not all the currently available systems rely on *ex vivo* cell manipulation, which concordantly leads to short T cell survival *in vivo* after infusion. *In vivo* artificial expansion systems would clearly circumvent this problem. Nevertheless active immunotherapy is not always the solution since sometimes in some patients, the T cells that could be potentially *in vivo* expanded no longer exist because they have been deleted, killed or anergised. Therefore a flexible system should be constructed in order to performed both adoptive and/or active immunotherapy depending on the patients requirements. Currently there is no comprehensive artificial Antigen Presenting Cell system (aAPC) for both effective *ex vivo* and *in vivo* antigen specific T cell expansion. Therefore in order to address this we have constructed a novel artificial nano-sized super para magnetic antigen presenting cell system (aAPC) capable of priming and expanding antigen specific T cells *ex vivo* and *in vivo*. As defined by the NIH, nanotechnology uses nanoscale injectable, targeted and traceable devices capable of important immunological/clinical functions. This nano-system was constructed using the latest generation of immuno liposomes, approved for *in vivo* human use since they are non-toxic, biodegradable, avoid rapid recognition by the reticulo-endothelial system, are safe in terms of size being 50 times smaller than average

cells at 100nm, have good stability and favourable pharmacokinetic behaviour for effective *in vivo* trafficking. We have coated these liposomes with an optimised number of MHC Class I / peptide complexes and a specific selected range of adhesion (anti LFA-1), early activation (anti CD28 and anti CD27), late activation (4-1BB) and survival receptors (anti CD40L) in the form of Fab antibody regions or monoclonal antibodies. We have made these immuno liposomes traceable since they carry fluorescent lipids and iron oxide super para magnetic nano particles or spios of 13nm size, which make them traceable *in vivo* using fluorescence and/or by Magnetic Resonance Imaging (MRI). The super para magnetic liposomes are also able to facilitate their own focusing to specific organs, tumour sites or body areas by applying external magnetic attraction. Production of this nano immune liposome system in a ready to use form is achievable in less than 48 hrs and viable for at least 7 days. After *ex vivo* stimulation with this artificial nano system using CMV pp65 as a model antigen, we have established successful expansions with high T cell numbers (55 to 200 fold) in CMV positive individuals, which are superior when compared with other systems such as peptide pulsed DCs, which are one of the standard methods currently used, coated Daudi cells, magnetic commercial beads and modified tetramers. The T cells are fully functional in terms of degranulation and production of cytokines when specifically challenged. They express predominantly effector-memory and memory phenotypes. We have demonstrated by double fluorescent staining that these liposomes activate T cells directly in an antigen specific fashion and also semi-directly by being incorporated on the surface of the natural APCs in a similar manner as exosomes. When tested in naïve individuals, this nano system was also capable of accomplishing initial low levels of T cell priming without help of any adjuvants. In conclusion, we have generated an efficient artificial APC, which embodies a powerful, controllable and superior approach with enormous potential for cancer nanotechnology and T cell immunotherapy for use either *in vivo* or *in vitro*.

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TABLE OF CONTENTS

Abstract	4
Acknowledgements	6
Table Of Contents	8
Appendix	18
Bibliography	19
List Of Figures	20
Abbreviations	24

CHAPTER 1

INTRODUCTION

1	HUMAN IMMUNOLOGY.....	28
	The Immune System.....	28
	1 The Innate Immune System.....	28
	2 The Adaptive Immune System.....	32
	2.1 Antigen presenting Cells.....	32
	2.1.1 Antigen Presenting Cell Origin	33
	2.1.2 APC Development And Antigen Presentation	37
	2.1.2.1 HLA Molecules	39
	2.1.3 Antigen Processing And Presentation Pathways	40
	2.1.3.1 Cross Presentation	40
	2.1.3.2 Class I presentation Pathway	41
	2.1.3.3 Class II Presentation Pathway	43
	2.1.3.4 Non Classical Antigen Presentation Pathways	43
	a) Extra cellular Antigen Processing And Presentation	43
	b) Exosomes (Semi Direct Antigen Presentation Pathway)	44
	2.1.4 Artificial Antigen Presenting Cells	45
	2.2 T Cells.....	46
	2.2.1 T Cell Origin	46
	2.2.2 T Cell Development	47
	2.2.2.1 TCR Gene Rearrangement And Phenotype Acquisition	47
	2.2.2.2 TCR Repertoire Selection And Self Tolerance Induction	50
	2.2.2.3 TCR Triggering	52
	2.2.2.4 T Cell Homeostasis	53
	2.3 APC – T Cell Interaction.....	56
	2.3.1 T Cell Antigen Recognition	56

2.3.2 T Cell – APC Reciprocal Modulation	57
2.3.3 T Cell Activation	57
2.3.3.1 Immunological Synapse	57
a) Costimulatory Signals	60
1) Adhesion Molecules	60
2) Early Activation Signals	61
3) Late Activation And Survival Signals	64
4) Expansion Signals	65
5) Cytokines	65
2.4 T Cell Mediated Killing.....	66
2.4.1 T Cell Affinity, Avidity and Recognition Efficiency	68
 2 IMMUNOTHERAPY.....	 69
2.1 T Cell Mediated Immunotherapy.....	70
2.1.1 T Cell Adoptive Immunotherapy	72
2.1.2 T Cell Active Immunotherapy	72
 3 TUMOR AND PATHOGEN ESCAPE.....	 73
3.1 Immune Evasion Strategies.....	74
3.1.1 Class I And Class II Down Regulation	74
3.1.2 Class I And Class II Total Loss	74
3.2 Immune Suppression Strategies.....	75
3.2.1 Immunosuppressive Receptors And Cytokines	75
3.3 Immune Tolerance Mechanisms.....	75
 4 NEW THERAPEUTIC TECHNOLOGIES.....	 77
4.1 Liposome Technology.....	77
4.2 Nanotechnology.....	78

5	SUMMARY AND AIM: DEVELOPMENT OF A NOVEL NANOTECHNOLOGY BASED ARTIFICIAL APC FOR T CELL MEDIATED IMMUNOTHERAPY.....	79
6	AIMS AND OBJECTIVES.....	85

CHAPTER 2

MATERIALS AND METHODS

2.1	INTRODUCTION.....	86
2.2	IMMUNOLIPOSOMES.....	87
2.2.1	Preparation Of Immunoliposomes	87
2.2.2	Chromatographic Purification	92
2.2.3	Concentration, Filtration And Storage	92
2.2.4	Dot Blot	92
2.3	MHC/PEPTIDE COMPLEX SYNTHESIS.....	93
2.3.1	Preparation Of MHC Class I Monomers	93
2.3.2	SDS-PAGE Gel	96
2.3.3	BCA Protein Quantification Assay	96
2.3.4	MHC/Peptide Complex Refolding	97
2.3.5	Biotinylation And Tetramer Synthesis	98
2.3.6	Native Gel	98
2.4	GENERATION OF FAB REGIONS.....	100
2.4.1	Antibody Purification	100
2.4.2	Antibody Digestion	103
2.5	STIMULATION CONDITIONS.....	103
2.5.1	Samples	103
2.5.2	Peripheral Blood Mononuclear Separation	106

2.5.2.1	Cell Counting And Viability	106
2.5.2.2	Cell Cryopreservation	106
2.5.2.3	Thawing Cryopreserved Cells	107
2.5.3	CD8 Positive T Cell Sorting	107
2.5.4	aAPC <i>Ex Vivo</i> Stimulation	109
2.5.5	T Cell Antigen Specific Sorting	109
2.5.6	Dendritic Cell Cultures	111
2.6	FUNCTIONAL ASSAYS.....	115
2.6.1	T2 Cells	115
2.6.2	Tetramer Staining And Flow Cytometry	115
2.6.3	Phenotypic T Cell Characterization	116
2.6.4	IFN- γ Intracellular Staining	116
2.6.5	Degranulation Assay	117
2.7	<i>IN-VIVO</i> MOUSE MODEL.....	118
2.8	MISCELLANEOUS.....	118
2.8.1	Desalting Columns	118
2.8.2	MRI Equipment	119

CHAPTER 3

CONSTRUCTION OF A SUPER PARA MAGNETIC

ARTIFICIAL NANO APC

FOR ACTIVE AND ADOPTIVE IMMUNOTHERAPY

3.1	INTRODUCTION.....	121
3.2	IMMUNOLIPOSOMES.....	131
3.3	COATING LIPOSOMES WITH MHC CLASS I	
	MONOMERS, mABS AND FAB REGIONS.....	134
3.3.1	Confirmation Of Liposome-MHC Class I Monomer Attachment	142
3.3.2	Confirmation Of Liposome-mAB and Fab Regions Attachment	145
3.4	PRODUCTION OF FLUORESCENT AND SUPER PARA	
	MAGNETIC aAPCs.....	147
3.4.1	Fluorescent Liposomes	147
3.4.2	Super Para Magnetic Liposomes (Magnetoliposomes)	149
3.5	CONCLUSIONS.....	153

CHAPTER 4

aAPC FOR ADOPTIVE IMMUNOTHERAPY

4.1	INTRODUCTION.....	154
4.2	T CELL EXPANSION IN A MEMORY SETTING (CMV POSITIVE INDIVIDUALS).....	157
4.2.1	T Cell Function: Expanded T Cells Via aAPCs Retain Degranulation And Cytokine Production	168
4.2.2	Phenotype Of Expanded T Cells	172
4.2.3	T Cell Antigen Specific Sorting	174
4.2.4	Proposed aAPC Mechanisms Of Action	174
	4.2.4.1 aAPC Direct Activation	176
	4.2.4.2 aAPC Semi-Direct Activation	177
4.3	T CELL PRIMING IN NAÏVE (CMV NEGATIVE) INDIVIDUALS.....	180
4.4	DISCUSSION.....	184
4.5	CONCLUSION.....	187

CHAPTER 5

aAPC FOR ACTIVE IMMUNOTHERAPY

5.1	INTRODUCTION.....	189
5.2	ASSESSMENT OF <i>IN VIVO</i> aAPC PRESENCE BY FLUORESCENT MICROSCOPY.....	194
5.3	MRI <i>IN VIVO</i> aAPC STUDIES.....	203
5.4	CONCLUSIONS.....	211

CHAPTER 6

CONCLUSIONS

6.1	SUMMARY AND GENERAL CONCLUSIONS.....	212
6.2	FUTURE PERSPECTIVES.....	214
6.2.1	Liposomes Interior	214
6.2.2	Tumour Antigen Liposomes	214
6.2.3	Class II aAPCs	214
6.2.4	aAPCs For Negative Vaccination	215

APPENDIX

APPENDIX 1: Publications And Presentations.....216

a) Publications	216
b) Published Abstracts	216
c) Oral Presentations	217

APPENDIX 2: Awards.....219

BIBLIOGRAPHY

BIBLIOGRAPHY.....220

LIST OF FIGURES

CHAPTER 1

FIGURE 1.1: INNATE IMMUNE RECOGNITION SYSTEM	31
FIGURE 1.2: APCs ORIGIN	34
FIGURE 1.3: GENERATION OF DCs	36
FIGURE 1.4: T CELL DIFFERENTIATION	55
FIGURE 1.5: T CELL ACTIVATION	59
FIGURE 1.6: B7 LIGANDS	62
FIGURE 1.7: B7 LIGANDS	63

CHAPTER 2

FIGURE 2.1: PREPARATION OF IMMUNOLIPOSOMES	88
FIGURE 2.2: MINI EXTRUDER	89
FIGURE 2.3: SCANNING ELECTRON MICROSCOPE IMAGE OF LIPOSOMES	89
FIGURE 2.4: SEPARATION OF INCLUSION BODIES BY SDS-PAGE	95
FIGURE 2.5: BCA PROTEIN ASSAY	95
FIGURE 2.6: MHC CLASS I MONOMER FPLC PROFILE	99
FIGURE 2.7: BIOTINYLATED MHC CLASS I MONOMER FPLC PROFILE	99
FIGURE 2.8: ASSESSMENT OF BIOTINYLATION EFFICIENCY BY NATIVE GEL	101
FIGURE 2.9: ANTIBODY PURIFICATION GEL	102

FIGURE 2.10: ANTIBODY ENZYMATIC DIGESTION	104
FIGURE 2.11: SEPARATION OF FAB REGIONS BY SDS-PAGE	105
FIGURE 2.12: CD8 + T CELL SELECTION	108
FIGURE 2.13: FLUORESCENT IMMUNO LIPOSOMES	110
FIGURE 2.14: T CELL ANTIGEN SPECIFIC SORTING	112
FIGURE 2.15: DC MATURATION	114
FIGURE 2.16: IN VIVO MOUSE MODEL	120
FIGURE 2.17: MRI EQUIPMENT FOR ANIMAL STUDIES	120

CHAPTER 3

FIGURE 3.1: ARTIFICIAL APC MODEL	133
FIGURE 3.2: CYSTEINE POSITIONS IN THE MONOMER STRUCTURE	135
FIGURE 3.3: CYSTEINE POSITIONS IN THE ANTIBODY AND FAB REGIONS STRUCTURE	136
FIGURE 3.4: LIPOSOME CL-4B CHROMATOGRAPHIC COLUMN PROFILE	138
FIGURE 3.5: LIPOSOMES, MHC MONOMERS, ANTIBODIES AND FAB REGIONS CL-4B CHROMATOGRAPHIC COLUMN PROFILE	139
FIGURE 3.6: ANTI CLASS I ANTIBODY RECOGNITION SITES	140
FIGURE 3.7: LIPOSOME - T CELL ANTIGEN SPECIFIC BINDING	141
FIGURE 3.8: CONFIRMATION OF MHC PEPTIDE COMPLEX- LIPOSOME ATTACHMENT BY DOT BLOT	143
FIGURE 3.9: CONFIRMATION OF AB AND FAB - LIPOSOME ATTACHMENT BY DOT BLOT	144
FIGURE 3.10: CO-ACTIVATORY SIGNALS EFFECT ON T CELL EXPANSION	146
FIGURE 3.11: FLUORESCENT LIPOSOMES	148
FIGURE 3.12: MRI <i>IN VIVO</i> TRACEABLE aAPC	150
FIGURE 3.13: SPIOS-LIPOSOME LOADING IMPROVEMENT	152

CHAPTER 4

FIGURE 4.1: DOSE DEPENDENT LIPOSOME / T CELL EXPANSION	159
FIGURE 4.2: aAPC MEDIATED T CELL EXPANSION	160
FIGURE 4.3: ANTIGEN SPECIFIC T CELL EXPANSION BY DCs	161
FIGURE 4.4: T CELL EXPANSION USING COMMERCIAL BEADS	162
FIGURE 4.5: aAPC - T CELL ANTIGEN SPECIFIC BINDING	164
FIGURE 4.6: aAPC - T CELL BINDING	165
FIGURE 4.7: CD8 + T CELL SELECTION	166
FIGURE 4.8: aAPC / CD8 DIRECT INTERACTION	167
FIGURE 4.9: T CELL FUNCTION: CORRELATION BETWEEN TETRAMER STAINING AND IFN- γ INTRACELLULAR STAINING	169
FIGURE 4.10: T CELL IFN- γ INTRACELLULAR STAINING	170
FIGURE 4.11: T CELL FUNCTION	171
FIGURE 4.12: PHENOTYPE OF aAPC EXPANDED TETRAMER POSITIVE CELLS	173
FIGURE 4.13: T CELL ANTIGEN SPECIFIC SORTING	175
FIGURE 4.14: aAPC BINDING TO NATURAL APCs	179
FIGURE 4.15: T CELL PRIMING IN NAÏVE CMV NEGATIVE INDIVIDUALS	182
FIGURE 4.16: T CELL PRIMING IN THE PRESENCE OF CD40L	183

CHAPTER 5

FIGURE 5.1: ARTIFICIAL NANOTECHNOLOGY AND SUPER PARA MAGNETIC APC	192
FIGURE 5.2: <i>IN VIVO</i> MOUSE MODEL	195
FIGURE 5.3: MOUSE PERIPHERAL BLOOD <i>IN-VIVO</i> PRESENCE	196
FIGURE 5.4: MOUSE LIVER AND HEART BLOOD VESSEL <i>IN VIVO</i> PRESENCE	197

FIGURE 5.5: MOUSE LUNG BLOOD VESSEL <i>IN VIVO</i> PRESENCE	198
FIGURE 5.6: MOUSE SPLEEN <i>IN VIVO</i> PRESENCE	199
FIGURE 5.7: aAPC <i>IN VIVO</i> PRESENCE	200
FIGURE 5.8: aAPC <i>IN VIVO</i> PRESENCE	201
FIGURE 5.9: MRI <i>IN VIVO</i> TRACEABLE aAPC	205
FIGURE 5.10: HIGH RESOLUTION MRI (T1)	209
FIGURE 5.11: aAPC MRI <i>IN VIVO</i> DETECTION	210

ABBREVIATIONS

aAPC	artificial Antigen Presenting Cells
ANRI	Anthony Nolan Research Institute
APC	Allo Phyco Cyanine
APCs	Antigen Presenting Cells
ATP	Adenosine Tri Phosphate
BD	Becton Dickinson
BMT	Bone Marrow Transplantation
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
cDNA	complementary Deoxyribo Nucleic Acid
CML	Chronic Myeloid Leukaemia
CMV	Cyto Megalo Virus
Cpm	Count per minute
CTL	Cytotoxic T Cell
CTLA-4	Costimulation T Lymphocyte Antigen 4
DA(s)	Dalton(s)
DC	Dendritic Cell
DLI	Donor Lymphocyte Infusion
DMSO	Di Methyl Sulph Oxide
DNA	Deoxyribo Nucleic Acid
EDTA	Ethylene Diamine Tetra acetic Acid
ELISA	Enzyme Linked Immuno-Sorbent Assay
EMF	Experimental Mean Fluorescence
ER	Endoplasmic Reticulum
FACS	Fluorescent Activated Cell Sorting
FCS	Foetal Calf Serum

FDA	Food and Drug Administration
FITC	Fluorescein Iso Thio Cyanate
FPLC	Fast Protein Liquid Chromatography
FSC	Forward Side Scatter
g	gram(s)
GMCSF	Granulocyte Macrophage Colony Stimulating Facto
GMP	Good Medical Practise
GvHD	Graft versus Host Disease
GvL	Graft versus Leukaemia
HCl	Chloride Acid
HLA	Human Leukocyte Antigen
HPLC	High Performance Liquid Chromatography
hr	Hours
HSCT	Haematopoietic Stem Cell Transplantation
ICAM	Inter Cellular Adhesion Molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPTG	Iso Propyl Thio-b-D-Galactoside
IS	Immunological Synapse
i.v.	Intra Venously
KDA(s)	Kilodalton(s)
M	Molar
mA	milli Amperes
mDC	monocyte-deriver Dendritic Cell
MFI	Mean Fluorescence Intensity
MHC	Mayor Histocompatibility Complex
mg	milligram(s)
μl	microliter(s)
mM	milli Molar
MRI	Magnetic Resonance Imaging

MS	Magnetic Separation
MW	Molecular Weight
MWCO	Molecular Weight Cut Off
NaCl	Sodium Chloride
nm	nanometre(s)
NMWL	Nominal Molecular Weight Limit
NTU	Nottingham Trent University
OD	Optical Density
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PE	Phyco Erythrin
PerCP	Peridin Chlorophyll Protein
RFH	Royal Free Hospital
RNA	Ribo Nucleic Acid
RPMI	Roswell Park Memorial Institute (culture media)
RT	Room Temperature
SC	Subcutaneously
SDS-PAGE	Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis
SPIOS	Super Para magnetic Iron Oxide nano particleS
SSC	Side Scatter
TAA	Tumour Associated Antigen
TAP	Transporter Associated with antigen Processing
TCR	T Cell Receptor
TEMED	N-N-N'-N' TEtra Methyl Ethylene Diamine
TIL	Tumour Infiltrated Lymphocytes
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
Treg	Regulatory T Cells
U	Unit

v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

1 HUMAN IMMUNOLOGY

The Immune System

The immune system comprises a complex army of specialized cells, cytokines, enzymes, antibodies, glands and organs that have the capability of identifying and destroying (in the majority of cases) foreign pathogens i.e. bacteria, virus, parasite and fungi as well as self cells that have become genetically abnormal, unstable or cancerous (Charles A. Janeway 2003). The immune system is not only able to eradicate diseases, but is able to “remember” and forms memory that enables a faster and improved response every time it reencounters antigens. The coordination and complexity of the immune system still amazes scientists. Unveiling and revealing its mechanisms has driven science and medicine to uncover many detail of interactions, important since manipulating the immune system correctly has and is helping us to live longer and better lives. Without the immune system, we would simply not survive for long in the outside world. The complexity of the immune system starts with the interaction between the so-called innate and adaptive immune systems.

1 The Innate Immune System

The innate immune system (also termed non-specific immune system) is our first line of defence against invading organisms. Even though it has been categorised as non-specific, its role in the organization of adaptive or more elaborate immune responses is essential and some recognition patterns by cells from the innate system do show specificity (Kopp and Medzhitov 1999). The innate immune system

consists of physical anatomical barriers as well as molecules and cells working together in order to protect us from pathogen attack. The physical anatomic barriers such as the skin and gastrointestinal and respiratory mucosal linings contain enzymes, secretions, saliva, acids and cilia that prevent pathogens from entering the body (Ouellette 1999; Krisanaprakornkit, Kimball et al. 2000). Examples of these are the fatty acids in sweat that inhibit the growth of bacteria, enzymes such as lysozyme and phospholipase found in tears, saliva and nasal secretions that can break down the cell wall of bacteria, the normal flora of the skin and gastrointestinal tract prevents the colonization of pathogenic bacteria by secreting toxic substances or by competing with pathogenic bacteria for nutrients or attachment to cell surfaces. However these primary defences can be penetrated and once a pathogen has entered, several secondary innate mechanisms enter in place and get activated in order to control the foreign agent. These internal innate defence mechanisms include the complement system (Frank and Fries 1991), which is able to lyse pathogens directly and recruit phagocytic cells, the coagulation system that apart from healing wounded tissues is also able to kill gram positive bacteria by releasing beta-lysine (a protein produced by platelets) and phagocytic cells that produce interferons, which prevent viral replication in cells (Durbin, Fernandez-Sesma et al. 2000), and interleukin 1 that induces fever and production of antimicrobial peptides by other cells (Bowie and O'Neill 2000).

In contrast to the adaptive immune system, which normally requires some time to react to a foreign pathogen, the innate system is armed with components that are constitutively present and ready to act at any moment. The cellular component of the innate immune system consists of neutrophils, macrophages, natural killer cells (NK) and eosinophils (Fearon and Locksley 1996). These cells are recruited to the site of invasion through danger (SOS) signals such as components released by bacteria, clotting system peptides, complement peptides and cytokines produced by local macrophages (Lee, Brummet et al. 2000). Once at the site of infection, innate immune cells have several different functional properties. Neutrophils (also called polymorphonuclear cells due to their lobed nuclei) phagocytose invading pathogens and kill them intracellularly by the use of “defensins” (Krisanaprakornkit, Kimball

et al. 2000), small proteins that include lytic enzymes such as elastase, cathepsine, lysozyme and oxidase among others. Circulating monocytes, which later mature into macrophages once in the tissues also kill intracellularly by phagocytosis, however they can also function as antigen presenting cells as explained in detail later on. NK cells can kill virus infected cells as well as tumour cells (Biron, Nguyen et al. 1999), and eosinophils possess proteins in granules that are effective in killing certain parasites. All four types of innate immune cells are able to recognise the Fc regions of certain type of antibodies (IgG and IgE) when attached to a specific antigen target, which induce activation of the cells (DeVries, Ran et al. 1999). Some of the innate immune cells such as phagocytic cells are able to recognise common pathogen but not human structures or conserved motifs called Pathogen-Associated Molecular PatternS or PAMPS (Bianchi 2006). These include bacterial lipopolysacarides (LPS), bacterial DNA and viral single stranded RNA among others. The innate immune cells recognise these motifs through Toll-like receptors (Kopp and Medzhitov 1999; Lien, Sellati et al. 1999), which make this recognition not entirely non-specific (fig 1.1).

It is important to highlight that pathogen infection does not necessarily mean disease as the immune system in most cases deals with most of the pathogens successfully. However sometimes when an undergoing effective and efficient immune response is taking place, part of the response mediated by cytokines, enzymes and toxins may produce collateral damage not only to healthy tissues but to the general well being of the person. An example of this is fever, which is a defence mechanism that affects the general condition that is intended however to kill pathogens by modifying the temperature.

In summary, an innate immune response is considered to be an immediate antigen “independent” response mediated by anatomical barriers, cells and components that do not produce immunological memory.

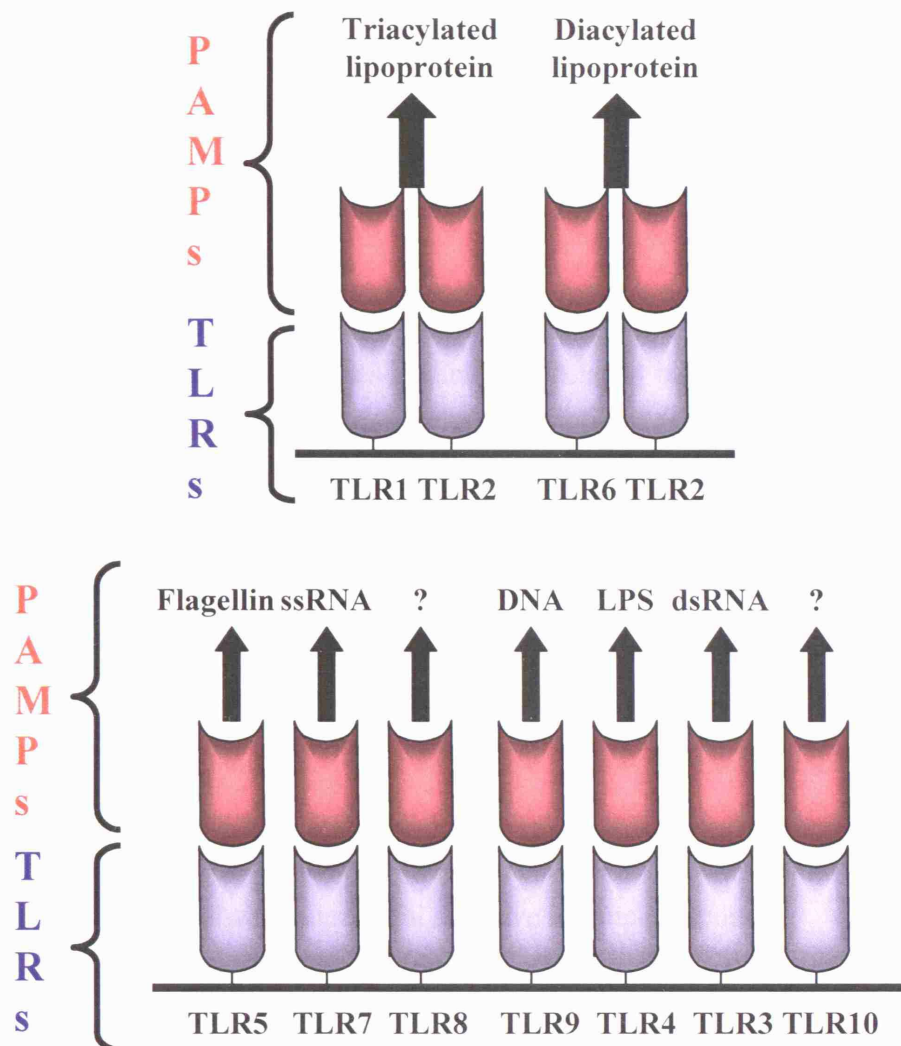


Figure 1.1.

INNATE IMMUNE RECOGNITION SYSTEM: Cells from the innate immune system recognise a series of PAMPs or pathogen-associated molecular patterns through Toll-like receptors (TLRs), which are shown above. Some of these PAMPs are mainly bacterial, fungal and viral products. Some are surface receptors whereas others are intracellular proteins released after destruction.

2 The Adaptive Immune System

When a pathogen overwhelms the innate immune system and the innate immune system cannot deal with it efficiently, the adaptive immune system is required. It is the innate system however who “decides” when and how that happens (Fearon and Locksley 1996; Fraser, Koziel et al. 1998). The adaptive immune system is a masterpiece of evolution and possesses a complexity of cell-cell interactions that will be detailed in the next sections. The characteristics of adaptive immune responses however, are that they are antigen specific and produce immunological memory. These types of responses are initially delayed but are more powerful and more rapid with subsequent encounters. The cells involved in adaptive responses are antigen presenting cells, T cells and B cells. These latter two types of cells are the effector arms of the adaptive immune response and represent respectively the so-called cellular and humoral (antibody mediated) branches of the immune response. Since their roles are incredibly useful in eradicating both pathogens and tumour cells, a comprehensive overview of these cells will be outlined next. Some of these cells can kill target cells accurately, whereas others can modulate and even suppress immune responses using receptors, co-receptors, cytokines and signals delivered during the formation of immunological synapses, which overall orchestrate a balanced and almost always successful adaptive immune response.

2.1 Antigen Presenting Cells

The link between the innate and the adaptive immunity is provided by antigen presenting cells (APCs). Natural APCs are responsible for the organisation of powerful cytotoxic responses but also required for accomplishing self tolerance. For this reason APCs are one of the most important cells in the immune system. They are responsible for T cell activation, expansion, survival and final T cell destination including T cell death. APCs have a central role since they are responsible for mounting and organising adaptive immune responses. Dendritic cells, the most powerful antigen presenting cells, were first identified in the epidermis by Paul

Langerhans in the 18th century. However it was not until 1990 when Steinman demonstrated their role in initiating primary immune responses (Steinman 1991) that APCs gained their reputation they have today as one of the main protagonists of the immune system. Since then APCs have become an unprecedented discovery and the centre of tumour immunology and immunotherapy. Therefore understanding how APCs mount and switch on and off immune responses holds the secret for accomplishing successfully tolerance to allografts, eradication of tumours and control of otherwise fatal infectious diseases.

Any nucleated cell in the body can function as an antigen presenting cell since all of them can present peptides by MHC molecules when infected or damaged, a phenomenon that also makes any MHC expressing cell a potential target for activated T cells. When a foreign antigen is presented at the cell surface by MHC molecules, the encoded message normally means death and the presenting cell (whether it is an APC under certain conditions or a target cell) is effectively killed. However in order to prime naïve T cells to form an effector clone, the MHC peptide complexes (even though essential) are not sufficient for T cell activation (Jenkins 1994). Co-activatory signals are also required. There are only three types of cells capable of delivering both MHC peptide complexes and co-activatory signals in sufficient numbers concurrently. They are the “professional antigen presenting cells”, which are B cells, macrophages and dendritic cells (DCs).

2.1.1 Antigen Presenting Cell Origin

APCs come from pluripotent haematopoietic stem cells in the bone marrow. Then lymphoid progenitors produce B cells and plasmacytoid dendritic cells, whereas monocyte-derived dendritic cells and the rest of dendritic cells derive from a common myeloid progenitor (figure 1.2). As mentioned previously, although B cells, macrophages and dendritic cells are equipped to mount immune adaptive responses, dendritic cells are the most powerful APCs in the human immune system since dendritic cells can uptake and process all proteins and antigens indiscriminately. It is thought that DCs may initiate all T cell responses *in vivo*. B

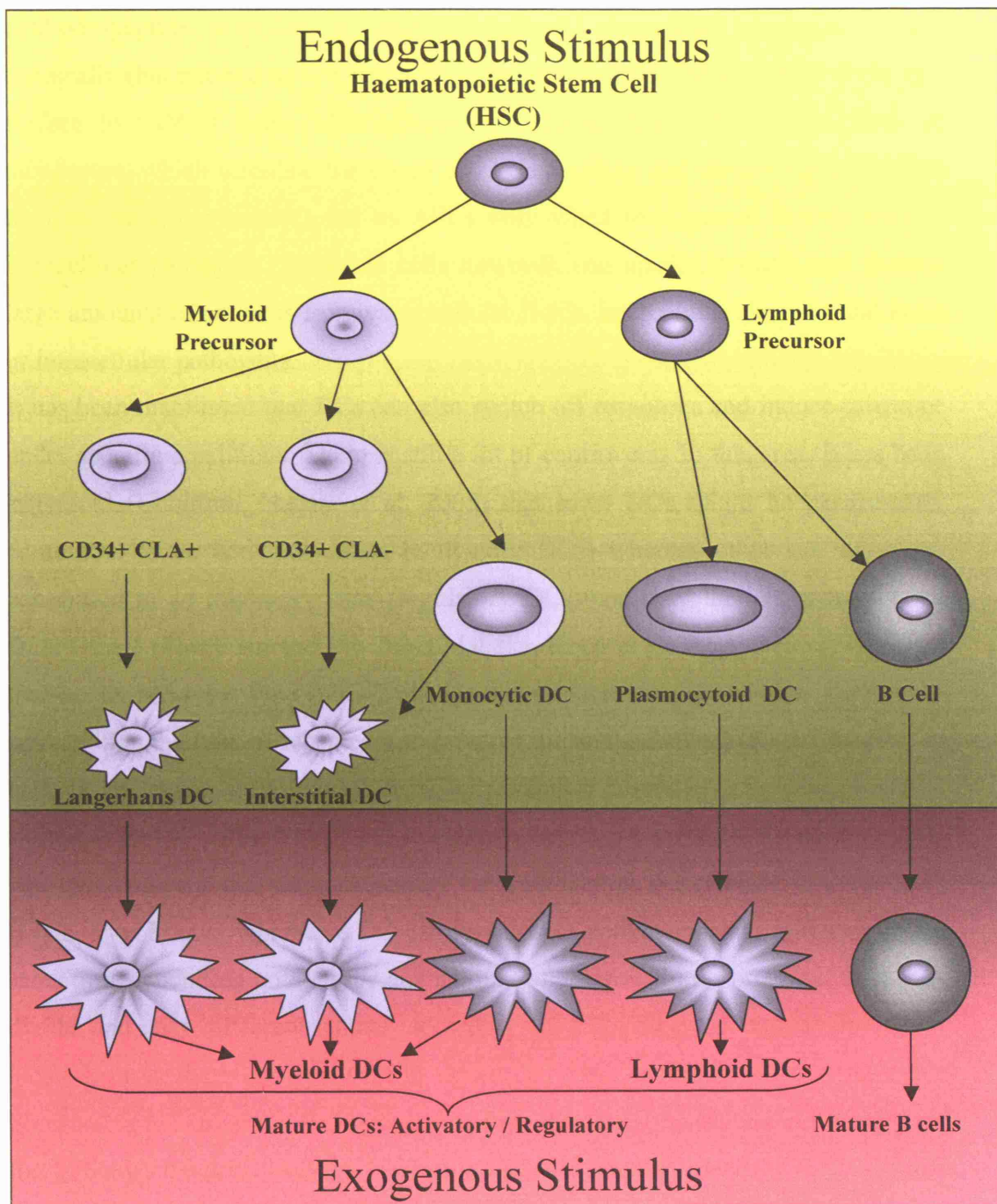


Figure 1.2.

APCs ORIGIN: DCs, B Cells and Monocytes share the same origin. The HSC generates a myeloid and lymphoid precursors independently of exogenous stimulus. The final maturation stage is antigen dependent and determines the fate of the APCs towards activatory or regulatory functions.

cells can only uptake the peptide recognised by its immunoglobulin receptor. The antibody-peptide complex is then internalised, degraded, processed, bound classically (but not exclusively) to MHC class II molecules and presented at the cell surface to CD4 T cells. Macrophages in contrast (the mature tissue form of monocytes, which circulate the blood until chemokines and danger signals attract them to particular tissues), act as APCs only when they cannot destroy certain intracellular pathogens. Dendritic cells however, can uptake, process and present large amounts of antigens from extra cellular fluids, intracellular proteins and extra or intracellular pathogens.

It has been mentioned that DCs can also switch off responses and induce tolerance under specific conditions. There is still a lot of controversy in this area. It has been suggested (Uchijima, Nagata et al. 2005) that some DCs might be permanently committed to an activatory state (activatory DCs) whereas other sub types are committed to an inhibitory state (regulatory DCs) and may include plasmacytoid DCs. Others (Shortman and Liu 2002; Mahnke, Knop et al. 2003) suggest however, that no matter what type of DC lineage a DC possesses, the critical step on its activation is the type of receptor that captures the antigen what will render a DC to become activatory or regulatory. It is well established however (Jenkins 1994) that an immature DC will induce T cell tolerance whereas the same DC upon maturation will then induce T cell activation and T cell modulation as explained in detail next. It is not yet known whether a DC can function as activatory and regulatory at the same time depending on the type and amount of antigen. So far however, DCs subdivide and take different names according to their receptor expression and/or localisation in the body as shown in figure 1.2 (MacDonald, Munster et al. 2002; Shortman and Liu 2002). Examples of this are the Langerhans cells in the skin and the follicular dendritic cells in the lymph nodes. Lymphoid dendritic cells express the α chain of the CD8 T cells and myeloid dendritic cells express the integrins CD11 b or c instead. Even though their myeloid and lymphoid names might imply different origins, their precursor is the exact same one progenitor from the bone marrow as shown in figure 1.2. Figure 1.3 describes the source, generation protocols and markers of some relevant subtypes of DCs.

Source	Generation Protocol	Markers
Adherent PBMCs: monocyte-derived DCs	IL-4, GM-CSF, Poly IC, TNF-α	HLA DR+, CD11+ TLR9-
CD34+ blood cells	TNF-α , GM-CSF	HLA DR+, CD11+ TLR9-
CD34+ blood cells	IL-3	HLA DR+, CD11- TLR9+
DC11c+ Blood Cells	None	HLA DR+, CD11c+ TLR9-
Langerhans cells from cord blood	None	HLA DR+, CD11c+ CD80+

Figure 1.3.

GENERATION OF DCs: DCs can be generated *ex-vivo* from different cell types. The generation of DCs from monocytes requires less amounts of blood precursors in comparison to the other subtypes. Therefore these cells are very often used as source of DCs.

2.1.2 APC Development And Antigen Presentation

An adaptive immune response (B cell or T cell mediated) is initiated when professional APCs present peptides picked up at the site of infection to T cells in the lymph nodes. Professional APCs in general are relatively long-lived, turning over at a slow rate. Once they leave the bone marrow, they migrate to the peripheral tissues where they survey the local environment and recirculate in the blood and regional lymph nodes. Immature dendritic cells carry receptors that recognise many common pathogen antigens such as bacterial cell wall proteoglycans, carbohydrate structures and lipopolysaccharides. These can be taken up by phagocytosis using receptors such as DEC 205 (Ebner, Ehammer et al. 2004) and Toll-like signalling receptors as previously shown in figure 1.1. These receptors then trigger activation of transcription factors such as NF κ B and a series of maturation events take place (see next). When immature dendritic cells engulf a pathogen, they degrade it intracellularly, process the peptides, enter a state of maturity, travel to the local lymph node and start mounting the immune response by presenting the peptides to T cells (Guermonprez, Valladeau et al. 2002). Dendritic cells also take up extra cellular material constitutively, including virus and bacteria particles by a receptor-independent pathway called macropinocytosis (Aderem and Underhill 1999). Again when the immature dendritic cells recover peptides from the periphery such as viral double-stranded RNA or bacterial unmethylated DNA motifs, DCs become rapidly activated by intracellular receptors such as TLR-9 (Barton, Kagan et al. 2006). This activates NF κ B, which triggers production of cytokines, interferon- α , interferon- γ and costimulatory molecules. Once the DCs are fully mature, they travel to the local lymph nodes to present the peptides to resting naïve T cells. When an APC becomes activated, it undergoes a series of important phenotypic changes; they change their cytokine secretion profile and become the directors and organisers of the following immune response. The main phenotypic changes of an activated or mature APC include high up-regulation and expression of MHC class I and II peptide complexes as well as a high expression of adhesion (ICAM-1, DC-SIGN) and costimulatory signals (B7.1, B7.2). ICAM-1 is one of the most important synergistic signals for T

cell activation during the immunological synapse formation (as described later on in detail). DC-SIGN (also described in detail later on) is known to be particularly important since it is one of the target receptors for HIV infection that not only produces immunosuppression by affecting APCs, but also allows virus dissemination and spreading to T cells when they interact with infected APCs (Engering, Geijtenbeek et al. 2002; Engering, Van Vliet et al. 2002). Once the APCs have matured, they are no longer able to uptake antigens by phagocytosis or macropinocytosis. Instead they acquire the ability to secrete a chemokine (DC-CK) that specifically attracts naïve T cells, which is one of the reasons why DCs are so powerful at activating naïve T cells. It is believed that DCs are the main or possibly only cells capable of priming naïve T cells. However if they do not encounter any pathogens and reach the end of their life span, they travel to the lymph nodes and die. Before dying however, some dendritic cells present self peptides to T cells that survived the repertoire selection (as explained below). Since these DCs were never activated, they do not present co-activatory signals and instead of inducing T cell activation, they induce tolerance on any T cell recognising them (Jenkins 1994; Johnson and Jenkins 1994).

Macrophages, which can also function as APCs, are scavengers of dead cells and form part of the first line of defence against infection. Macrophages have few or no MHC molecules and no co-activatory signals when resting. Unless they recognise pathogens unable to destroy by themselves, they will not contribute to adaptive immune responses. However when unable to destroy pathogens, they function and behave in a very similar way to dendritic cells. B cells on the other hand, although very efficient at presenting antigens, these have to be taken up via their immunoglobulin receptors and cannot process large or random peptides from the extra cellular fluid. However they are able to activate both CD4 and CD8 T cells when needed.

It is important to remember that T cells only recognise peptides when presented by specialised, polygenic and polymorphic molecules: the MHC molecules. MHC stands for Major Histocompatibility Complex, and in humans are codified by the HLA genes (Human Leukocyte Antigen). These molecules were so-named because

they were first described and identified by the potent immune responses they triggered in a transplant setting. MHC molecules are responsible for processing and presentation of peptides to T cells. Therefore we will review some of their most important characteristics.

2.1.2.1 HLA Molecules

The HLA genes are the most polygenic and polymorphic set of genes in the entire human genome. This is maintained as the polymorphism is favoured by evolution. The capacity that a species has to defend itself and mount immune responses against a plethora of pathogens is largely due to the number of MHC molecules present within a population. The more polymorphic our HLA gene repertoire is, the better, stronger and broader range of immune responses that we can mount. According to the ImMunoGeneTics (IMGT) project HLA database (www.ebi.ac.uk/imgt/hla/stats.html), up to October 2006, there were 2,510 class I and class II alleles divided as follows (courtesy of Dr Steven Marsh):

- ❖ 489 HLA class I A alleles
- ❖ 830 HLA class I B alleles
- ❖ 266 HLA class I C alleles
- ❖ 548 HLA class II DR alleles
- ❖ 112 HLA class II DQ alleles
- ❖ 148 HLA class II DP alleles

Individuals possess two either identical (homozygous) or different (heterozygous) copies for each HLA A, B, C, DR, DP and DQ alleles. These HLA alleles code for MHC molecules, which present peptides from the environment. Peptides bind to MHC molecules in two classic ways depending on where the peptide derives from. Peptides from the cytosol derived from intracellular pathogens are bound to MHC class I molecules and are recognised by CD8⁺ T cells, whereas extra cellular peptides internalised by vesicles are bound to MHC class II molecules and recognised by CD4⁺ T cells. DCs however can present extra cellular peptides via

class I molecules; a phenomenon called cross presentation and explained in detail next.

2.1.3 Antigen Processing And Presentation Pathways

As previously mentioned, the job of professional APCs begins with internalising pathogens, dead cells and extra cellular material including dying tumour cells by both a receptor-dependent mechanism (phagocytosis) and a receptor-independent mechanism (macropinocytosis). Whatever the mechanism, matter is internalised into intracellular vesicles where it will eventually be destroyed and/or processed and presented. APCs however, can also get infected and also need to present these pathogens in order to eliminate them. Internalised pathogens and infectious agents are not always destroyed and fighting these pathogens requires modified responses. These may be antibody production or boosting intracellular killing. Extracellular material that has been uptaken by APCs allows the loading of pathogen peptides classically onto MHC class II molecules, which will eventually activate CD4⁺ T cells. CD4 T cells then subdivide into T_H1 and T_H2 cells. T_H1 cells perform two essential functions: 1) activate macrophages to induce killing of the intravesicle pathogens they harbour, and perform 2) direct CD4 mediated killing of targets as explained in detail in section 1.1.2.4. T_H2 cells in contrast, activate B cells to produce specific antibodies. They also help prime of CD8⁺ T cells by delivering important survival signals either by cytokine production or by APC - T cell co-interaction (see next). In summary, when APCs capture peptides from the extra cellular compartment, these peptides are classically presented by MHC class II molecules and therefore will mount CD4 T cell responses. Dendritic cells however, can present extra cellular peptides by MHC class I molecules by a mechanism called cross presentation (Belz, Carbone et al. 2002).

2.1.3.1 Cross Presentation

The discovery that cross presentation was a feature of DCs was surprising as it was thought that DCs presented peptides by MHC class I molecules only when virus and

bacteria were replicating in their cytosol. Peptides from these cytosolic pathogens are normally processed and presented by MHC class I molecules, which are then recognised by CD8⁺ cytotoxic T cells. This action induces two reactions, first the APC will activate antigen specific T cells and an adaptive immune response is mounted, and second the infected APC will be killed upon interaction with these activated T cells specific for that antigen since the APC is infected after all. However DCs can also mount powerful class I - CD8 T cell responses without being infected or directly affected and even though DCs can mount class II responses, they preferentially mount class I cytotoxic responses when healthy. This feature was uncovered when CD8⁺ anti tumour responses were observed. Since tumour antigens are captured by APCs from the extra cellular compartment, these peptides should be therefore classically internalised by vesicles and presented to CD4 by MHC class II molecules. However DCs mount CD8⁺ potent anti tumour responses using class I molecules. Cross presentation (Guermonprez, Valladeau et al. 2002) is one of the reasons why dendritic cells are the most powerful APCs in the immune system as dendritic cells can present peptides via MHC class I molecules even when the peptides come from the extra cellular compartment rather than the cytosol. This phenomenon of cross presentation is a unique characteristic of dendritic cells.

Even though macrophages and B cells can load and present antigens via MHC class I molecules, their main mechanism of pathogen uptake is by endocytosis (phago or macropinocytosis) via intracellular vesicles, therefore macrophages and B cells preferentially mount class II immune responses.

2.1.3.2 Class I Presentation Pathway

Peptides from viruses and bacteria generated in the cytosol are presented by MHC class I molecules. These viral and bacterial peptides are actively (requires ATP) transported from the cytosol to the endoplasmic reticulum (ER) where they meet newly synthesised MHC class I molecules. Both peptide and MHC class I molecule must bind successfully to maintain a stable MHC peptide complex in the ER which

can then be transported via the secretory pathway to the cell surface. Two proteins TAP-1 and TAP-2 (Transporters associated with Antigen Processing) form an ER transmembrane heterodimer, which carries preferentially eight or more amino acid peptides from the cytosol into the ER to allow MHC class I / peptide binding (Monaco 1992). TAP deficient cells such as T2 cells (used as targets in our functional assays), fail to express MHC cytosolic peptide complexes on their surface since peptide transport from the cytosol to the ER is blocked. However T2 cells can present MHC peptide complexes containing self peptides resident within the ER (Barber, Whitelegg et al. 2004). These peptides however are often weak binders and although they reach the cell surface, they are not sufficiently strong to maintain stable complexes at the surface and are quickly recycled. We take advantage of this feature by feeding T2 cells with a strong binder such as the NLV pp65 CMV peptide. These peptides compete with the weak self peptides presented at the T2 cell surface and stabilise the MHC molecules at the surface (Barber, Whitelegg et al. 2004). In normal cells however, newly synthesised MHC class I molecules are retained in the ER unless they bind a peptide. Proteins from the cytosol enter a multicatalytic cylindrical protease complex called the proteasome where they are constantly degraded into small peptides (Niedermann, Geier et al. 1999). After this process the small peptides enter the ER via TAP-1 and 2. MHC class I molecules are formed by three proteins: an α heavy chain composed of three domains ($\alpha 1$, $\alpha 2$ and $\alpha 3$), a β_2 macroglobulin (β_2m) and the bound peptide. However before the peptide binds to an MHC molecule in the ER, the newly synthesised heavy chain is carried by calnexin, an ER resident chaperone. Once the heavy chain binds to the β_2m , calnexin is replaced by calreticulin (another chaperone), tapasin and Erp57. Tapasin makes a link between the MHC molecule and the TAP proteins to favour binding transported peptides. Erp57 is believed to work as a quality control mechanism during peptide binding. Once a peptide binds successfully to an MHC class I molecule, the complex is released and leaves the ER to be transported to the cell surface where it is presented to circulating CD8⁺ T cells and class I peptide presentation is accomplished.

2.1.3.3 Class II Presentation Pathway

Peptides presented by MHC class II molecules, which are composed of two glycoprotein chains (α and β), have different binding kinetics. They are not digested by the proteasome since they are encapsulated in intracellular vesicles (endosomes or lysosomes). Instead they are digested into small peptides by low pH activated enzymes (cathepsins) inside these acidic vesicles. MHC class II molecules are also synthesised in the ER. However peptide binding in the ER is prevented by a protein termed the MHC class II associated invariant chain (Ii). The Ii protein blocks the MHC class II molecule peptide groove preventing binding of any other peptides present in the ER. The Ii protein also directs the class II molecules into low pH vesicles that leave the ER and fuse with peptide loaded endosomes or lysosomes. Once fusion has occurred, cathepsins cleave the Ii protein leaving only a small Ii fragment still attached to the class II peptide binding groove. This remaining Ii peptide is called CLIP (class II associated invariant chain peptide), which is eventually displaced by catalysis by a protein called HLA-DM. This allows other peptides to bind in the groove. HLA-DM binds, removes and rebinds vesicle peptides constantly; a phenomenon termed peptide editing. Weak binders are easily removed by HLA-DM, ensuring that the final peptides bound to class II molecules are strong binders. The vesicles entrapping new MHC class II peptide complexes then fuse with the cell membrane where they are presented to CD4⁺ T cells.

2.1.3.4 Non Classical Antigen Presentation Pathways

a) Extra cellular Antigen Processing And Presentation

A pathway that involves antigen processing, loading and presentation occurring entirely outside the cell has been described for class II molecules (Santambrogio, Sato et al. 1999; Santambrogio, Sato et al. 1999). We have discussed how class II peptide complexes are normally processed in the previous section. However, some immature DCs express both MHC class II and HLA-DM complexes at the cell

surface. DCs secrete proteolytic enzymes capable of processing intact proteins into peptide antigens that bind to these MHC class II complexes previously mentioned, a phenomenon that results in MHC class II peptide processing, loading and presentation completely extracellularly. The relevance of this pathway in normal immune responses is still under investigation. However it is well described and it is likely to have an important role.

b) Exosomes (Semi Direct Antigen Presentation Pathway)

APCs possess several means of amplifying immune responses. When peripheral APCs uptake antigens from the periphery, they mature and travel to the lymph nodes. Since naïve T cells for a particular antigen can be as low as 1 in 1 million T cells (Blattman, Antia et al. 2002), a T cell needs to screen a huge number of APCs in order to find its peptide. In order to accelerate this process, APCs improve the peptide T cell screening by releasing vesicles called exosomes. Exosomes are vesicles around 30-100 nm size coated with MHC class I and class II peptide complexes and several costimulatory signals such as B7.1 and ICAM1 (Thery, Zitvogel et al. 2002; Segura, Amigorena et al. 2005). Exosomes have an endocytic origin, but are secreted into the extra cellular space in order to “share” and communicate with neighbour cells. Exosomes can function in very different ways. APCs release exosomes that bind to the surface of other APCs waiting in the lymph nodes. In doing so, many other APCs that did not encounter the antigen themselves are now ready to activate T cells in a so-called semi-direct presentation pathway (Herrera, Golshayan et al. 2004). Dendritic cell derived exosomes can stimulate immune responses *ex vivo* (Hsu, Paz et al. 2003) and *in vivo* (Lamparski, Metha-Damani et al. 2002; Andre, Chaput et al. 2004; Segura, Nicco et al. 2005). It is termed the semi-direct pathway of antigen presentation since it is still controversial whether exosomes secreted by mature APCs at the moment of the immunological synapses can act themselves as micro APCs directly (Thery, Duban et al. 2002). It has been proven however that they function bound on the surface of other DCs, therefore semi-directly. APCs communicate between themselves (cross-talk) in

order to mount effective responses and use of exosomes is one way in which they can achieve this. Many cells can release exosomes including tumour cells. These exosomes do not contain MHC peptide complexes or costimulatory molecules. Instead they carry only tumour antigens and they work differently. These tumour cell derived exosomes can be intercepted *in vivo* or artificially selected *ex vivo* to feed APCs in order to mount effective anti tumour responses by cross priming (Wolfers, Lozier et al. 2001). Exosomes are therefore important not only because of their mechanism of action, but also because as explained later on in chapter 4, they allow us to create artificial APCs which resemble exosome mechanisms of action during T cell activation and expansion.

2.1.4 Artificial Antigen Presenting Cells

Since APCs have a fundamental role in mounting, organising and suppressing immune responses, there has been a large drive to mimic their function in order to manipulate the immune system in instances where diseases such as cancer and viral infections have either overwhelmed, suppressed and evaded the immune system. Manufacture of artificial antigen presenting cells produces reagents with “off the shelf” availability. This avoids the need to recover and grow natural APCs from patients and/or donors, and also avoids the use of expensive and time consuming protocols, which are often neither reproducible nor reliable. Artificial APC systems are currently used to activate T cells in several ways, either polyclonally or with antigen specificity. Some have reached the clinical trial stage and are starting to revolutionise the way immunotherapy is delivered to patients. A comprehensive overview of the current artificial systems including advantages and disadvantages will be outlined in detail in chapter 3. However systems are divided into cell based and non-cell based aAPCs (Kim, Latouche et al. 2004; Oelke, Krueger et al. 2005), as listed below:

Cell Based aAPCs

- ❖ Transfected insect cells (Cai, Brunmark et al. 1996)

- ❖ Transfected animal cells (Latouche and Sadelain 2000; Schneck 2000)
- ❖ Transfected human cell lines (Maus, Thomas et al. 2002; Thomas, Maus et al. 2002)

Non-cell Based aAPCs

- ❖ Magnetic Beads (Lum, LeFever et al. 2001; Maus, Riley et al. 2003) (Oelke, Maus et al. 2003) (Schilbach, Kerst et al. 2005)
- ❖ Exosomes (Wolfers, Lozier et al. 2001; Thery, Duban et al. 2002; Hsu, Paz et al. 2003; Andre, Chaput et al. 2004; Segura, Amigorena et al. 2005; Segura, Nicco et al. 2005)
- ❖ Tetramers (Maile, Wang et al. 2001), Modified Tetramers and Multimers
- ❖ Tetramer + Soluble co-activatory cocktails in the form of antibodies

2.2 T Cells

2.2.1 T Cell Origin

Lymphopoiesis is the term given to the generation and production of lymphocytes (B cells and T cells). Since T cells represent one of the most powerful killing machineries in the adaptive immune system, and are also the centre of many immunotherapeutic approaches, we will concentrate on the study of T cells rather than B cells. T cells are so-named because they complete their development in the thymus. The thymus is a specialized lymphoid tissue and one of the “central lymphoid tissues”. Central lymphoid tissues (Bone Marrow and Thymus) are the origin source for lymphocytes. Immature precursors from the bone marrow have the capacity to become B cells, NK cells or T cells. It is the thymus microenvironment that programs the cells to become T cells.

2.2.2 T Cell Development

In order to provide protection against the multitude of known and potentially unknown pathogens we can encounter, T cells must be immensely variable in their antigen specificity. However, it would be impossible to have a single gene encoding a T cell receptor with a given antigen specificity since there would be more genes than there are in the entire genome. Even if that were the case, there is not the available space inside the body to store and expand these unlimited single antigen specific T cells. Therefore the immune system has developed two interesting features; cross reactivity, also called T cell plasticity (Selin, Cornberg et al. 2004; Selin and Welsh 2004), which is the ability of a single T cell to recognise more than one target ($\sim 10^5$ MHC peptide different complexes) (Mason 1998), and gene rearrangement, which is the ability of T cell precursors to rearrange a limited number of genes in order to create a virtually unlimited number of new ones. These new sequences then produce the wide variety of T cell receptors as explained next.

2.2.2.1 TCR Gene Rearrangement and Phenotype Acquisition

T cells can be separated according to the composition of their receptors: These are termed either $\alpha:\beta$ T cells or $\gamma:\delta$ T cells. Our main interest concerns the $\alpha:\beta$ subtype not only because they form the majority of the T cell pool (95%), but also because they further subdivide into CD8 and CD4 T cells, which have major roles in adaptive immune responses. Whatever the subtype however, $\alpha:\beta$ T cells recognise antigens due to a specific amino acid sequence at the antigen binding site of the T cell receptor. This antigen binding site is made up from a combination (gene rearrangement) of regions, which are encoded on the receptor protein gene sequence as sets or segments. It is a particular rearrangement of these gene segments that makes a unique combination and a unique T cell receptor. The $\alpha:\beta$ T cells are made from an α chain and a β chain. The α chain locus in chromosome 14 contains 70 to 80 V (variable), 61 J (joining) and a single C (constant) gene segments called $V\alpha$, $J\alpha$ and $C\alpha$ respectively. The β locus in chromosome seven, which rearranges first

than the α locus, contains 52 V, 6 or 7 J, 2 D (diversity) and two C gene segments called V β , J β , D β and C β respectively. These rearrangement events altogether can potentially generate a total T cell diversity of $\sim 10^{18}$ different T cells in the thymus. As previously mentioned, the β chain genes rearrange first, with the D β gene segments rearranging to J β gene segments. This newly arranged DJ β segment rearranges then to a V β gene segment. Once a successful VDJ β chain segment has been rearranged, a surrogate α chain is attached to the β chain in order to form the pre-T cell receptor that will be expressed on the surface of the T cell. This process triggers the co-expression of CD4 and CD8 co-receptors as well as the stop signal for further β chain rearrangements. These events make the CD8-CD4 double positive T cells enter cell division and only then, the α chain starts to rearrange in a very similar way. Until this point, T cell development occurs completely independent of antigens; from then on the rest of the development decisions will depend on interactions between the T cell receptor and MHC/peptide molecules presented in the thymus. The ligand for T cell receptors always involves peptides presented by MHC molecules, therefore the highest variability of the T cell receptor is found in the peptide binding area and is determined mainly by the combination of the sequence regions encoded by V, D and the J segments. These are further modified by P and N nucleotide additions at the junctions of all rearranged α and β genes. The antigen binding TCR site encoded by the V, D and J segments is divided into three complementary determining or CDR regions (Bentley and Mariuzza 1996). CDR3 is where the TCR diversity focuses since it makes 50% of the contact with the target peptide, whereas CDR1 and CDR2 bind to the periphery of the peptide surrounded by the MHC molecule. The periphery of the receptor is relatively conserved.

As well as TCR generation during T cell maturation, other T cell markers start to be expressed. When T cell bone marrow precursors arrive at the thymus via its cortex, they are cell surface negative for CD4 and CD8 molecules, markers that determine their individual functional properties. At this stage they are called "double negative T cells". As soon as pre-T cell receptors (which are basically β chains + surrogate α chains) start to be expressed on the T cell surface, T cells express both CD4 and

CD8 receptors ("double positive T cells"). It is following T cell final selection (section 2.2.2.2) when T cells lose either CD4 or CD8 receptors depending on which MHC class I or II peptide complex they were able to recognise, that they form "single positive T cells", which are ready to leave the thymus via its medulla.

As previously mentioned, there is a minority of T cells (1 to 5%) that bear T cell receptors composed of one γ chain and one δ chain ($\gamma:\delta$ T cells). These are also called unconventional T cells due to their smaller proportion in the body. They come from the same T cell precursor and their final protein chains are also generated by gene rearrangements (Carding and Egan 2002). The function of $\gamma:\delta$ T cells is largely unclear. Many of their ligands are also unknown and at the moment is not even clear whether they can recognise antigens bound to MHC peptide complexes or whether they act more like antibodies recognising entire antigens. They are considered to be MHC independent, peptide independent and the minority within the T cell population. However $\gamma:\delta$ T cells are rapidly activated upon pathogen recognition in several epitheliums and it seems that they play a role in tumour surveillance and killing (Girardi 2006). $\gamma:\delta$ T cells are commonly subdivided into V δ 1 and V δ 2 since in humans, only two δ genes, V δ 1 and V δ 2, are commonly used (De Rosa, Andrus et al. 2004). V δ 1 T cells are less frequent, comprising up to 10% of all $\gamma:\delta$ T cells and predominate at epithelial sites. However they are capable of recognising and killing malignant cells and viral infected cells in what is thought to be a TCR-independent fashion (Lamb and Lopez 2005). These V δ 1 T cells express receptors for double-stranded RNA, which is produced during viral replication and some other receptors found on NK cells. V δ 2 subsets on the other hand, circulate in blood and are thought to respond to microbial pathogens. Even though DCs are not required for their expansion, their response is enhanced by association with DCs (Eberl, Jomaa et al. 2004). $\gamma:\delta$ T cells preferentially migrate to the epithelia of the skin, reproductive, respiratory and gastrointestinal tracts where they can comprise up to 50% of T cells, whereas $\alpha:\beta$ T cells recirculate between peripheral lymph nodes and blood. $\gamma:\delta$ T cells are highly concentrated in the epidermis, they display uniform receptors that recognise single antigens (Jameson, Ugarte et al. 2002). When the skin is cut or damaged, epithelial cells release

antigens recognised by $\gamma:\delta$ T cells, which then get activated and produce growth factors that lead to healing of the wound. $\gamma:\delta$ T cells express NKG2D, which is a potent activatory receptor also found on NK and $\alpha:\beta$ T cells. Therefore $\gamma:\delta$ T cells are theoretically capable of recognising all NKG2D ligands such as MIC A, MIC B and RAET1 among other stress induced receptors, which will eventually lead to cytotoxic $\gamma:\delta$ T cell functions following recognition.

A T cell precursor can start rearranging β , γ or δ chains at the same time, α chains only start rearrangements once a β chain has rearranged successfully. It is not exactly known what makes a T cell precursor to produce an $\alpha:\beta$ T cell rather than a $\gamma:\delta$ T cell or vice versa. It might be a matter of random natural selection. A cell that rearranges a β chain will produce an $\alpha:\beta$ T cell, whereas a $\gamma:\delta$ T cell needs to rearrange both chains successfully at the same time in the same cell. However both $\alpha:\beta$ and $\gamma:\delta$ pre-receptors can be expressed at the same time by the same cell. If it receives a signal via the $\alpha:\beta$ pre-receptor, the cell will commit to that lineage, whereas a signal received via the $\gamma:\delta$ pre-receptor will commit the cell to the other lineage (Pennington, Silva-Santos et al. 2005). The details of this latest decision are not yet completely understood.

2.2.2.2 TCR Repertoire Selection And Self Tolerance Induction

Gene rearrangements produce incredibly variable antigen specificities. These are specific not only for foreign pathogens but can also recognise self peptides. Once gene rearrangements have produced a T cell receptor on the surface of the T cell, the T cell receptor is tested against self-peptides presented in the thymic environment by bone marrow derived dendritic cells (mainly) and thymic epithelial cells. This testing is performed to eliminate TCRs that could potentially recognize the body as foreign and therefore initiate a damaging autoimmune response. This ensures self tolerance is accomplished while a repertoire of useful T cells is selected. It is then the specificity and affinity of the T cell receptor for these self ligands that will determine the fate of each particular T cell. If the T cell receptor cannot bind self MHC peptide complexes presented in the thymus, the T cell will be

eliminated since it is pointless keeping a T cell unable to recognize self MHC complexes, which eventually will present foreign peptides in the future. Therefore these T cells unable to mount immune responses are eliminated. Many T cells cannot recognise MHC complexes because the genes for the T cell receptor α and β chains segregate independently from those of the MHC molecules. Genes for the MHC molecules also happen to be the most polymorphic in the human genome. Therefore many T cell receptors fail to recognize MHC peptide complexes.

T cell receptors that are able to bind self MHC peptide antigens moderately, receive a “positive selection” signal, enabling T cells to survive and continue maturation. These T cells are capable of recognizing own MHC peptide complexes, which means that they will be able to recognize foreign peptides when bound to self MHC molecules. However, the T cell-self peptide recognition is not strong enough to cause autoimmunity. T cell receptors that recognise self MHC peptide complexes strongly are also deleted to avoid autoimmunity, process called “negative selection”. So unless T cells receive a positive selection signal, the fate of the T cells is death. Approximately 98% T cells die in the thymus, which reflects the severe and intense screening they undergo. However T cells that survive positive and negative selection form a T cell repertoire that becomes the strongest arm of the adaptive immune response. It is important to mention that not all of self peptides are presented in the thymus during negative selection, for example some self antigens are found in the body only after puberty. Therefore a few T cells could find their targets after leaving the thymus as mature T cells and cause autoimmunity. This can happen, but normally other tolerance mechanisms take place such as the lack of co-activatory molecules in normal tissues, a phenomenon that renders T cells anergic (Jenkins 1994). Other anti-autoimmunity mechanisms are the antigen encapsulation of organs as in entrapped organs such as eyes. But probably one of the most important mechanisms to avoid autoimmunity is performed by immature peripheral dendritic cells that did not encounter pathogens and reached the end of their life span. When this happens, immature DCs travel to the lymph nodes and present self peptides to T cells that survived the repertoire selection. Since these DCs were never activated, they do not present co-activatory signals, which induce anergy and

tolerance on any T cell recognising these self peptides (Jenkins 1994; Johnson and Jenkins 1994).

As mentioned previously, following positive selection, T cells lose either the CD4 or CD8 receptors. T cells then leave the thymus and migrate to peripheral lymph nodes as single positive CD4⁺ or CD8⁺ T cells recirculating in the blood and the lymph nodes many times until they find their targets. CD4 T cells are mainly programmed to become cytokine producing cells although they are perfectly capable of killing, whereas CD8 T cells are programmed to become almost exclusively cytotoxic T cells.

2.2.2.3 TCR Triggering

Antigen recognition by T cells is one of the best-studied phenomenon. However the intracellular chain of events that follows antigen recognition by T cells is still controversial. P. Anton van der Merwe et al. discussed in *Nat Immunology* (2002) whether “T cell receptors do it alone” and nowadays there are still two main conflicting models: the kinetic-segregation model (Davis and van der Merwe 2006) and the heterodimerization model (Trautmann and Randriamampita 2003). The fundamental question is whether TCR triggering itself is co-receptor dependent or independent. Soluble factors, co-receptors and cell-cell interaction can either up (CD28, IL-2) or down (CTLA-4, IL-10) regulate the response. However the kinetic segregation model postulates that TCR triggering itself is independent of any of these external cues. In other words, for TCR triggering, there is no need for any prior TCR dimerization, CD8 or CD4 co-receptors recruitment or any other co-factor. It stipulates that the TCR is involved in scanning and recognition events prior to the immunological synapse formation. These areas of TCR interaction are called “close contact zones” and could occur on any part of the T cell membrane. Co-receptors do not participate in the formation of these zones and they are excluded from these very small contact zones. Since bulky enzymes such as dephosphatases are also excluded, and since TCR phosphorylation induces TCR triggering, the TCR becomes temporarily phosphorylated in the close contact zones

and some TCR triggering occurs. If antigen recognition is successful, following TCR triggering, downstream signals are initiated, co-receptors then participate and enhance signalling and the immunological synapse is formed. If TCR recognition is not successful, the TCRs leave the close contact zones and they are quickly dephosphorylated in order to avoid downstream signalling following TCR triggering. The kinetic-segregation model implies that phosphorylation of the TCR itself triggers the TCR even in the absence of ligand engagement. The segregation model accepts that TCR dimerization can also be a mechanism to trigger the TCR, tetramers for instance are believed to follow this pattern. However the kinetic segregation model supports the idea that a single TCR can be triggered in a co-receptor independent manner. This is further supported by others (Irvine and Davis 2002) showing that a single MHC peptide complex is sufficient to trigger the T cell receptor. The heterodimerization model on the other hand supports the idea that TCR triggering occurs only when the TCR and co-receptors bind to the same MHC peptide complex. Since T cell triggering is observed in animal models lacking co-receptors, it seems that co-receptors are very important to enhance TCR triggering, form stable immunological synapses and up or down modulate end-point responses, however they are not essential to trigger the TCR itself.

2.2.2.4 T Cell Homeostasis

T cells mature in the thymus as described previously. They leave the thymus to populate peripheral lymph nodes and circulate in the blood. The thymus slows down and contracts with time, although T cell production continues throughout life even after puberty when the T cell repertoire has been established. T cell immunity can be sustained in the absence of the thymus and T cell numbers are maintained through division of mature T cells outside the thymus to retain homeostasis. Once cytotoxic T cell responses are mounted as part of desirable immune responses, a T cell clonally expands 10 to 100 fold above its initial frequency reaching frequencies up to 70% to 80% of circulating cells. Therefore different mechanisms enter into place in order to control the level and the extension of T cell proliferation and

survival. DCs are responsible for regulating normal immune responses depending on the phenotype that they have induced on the T cells during activation. Terminal effector T cells for instance, are committed to kill relevant targets and programmed to die afterwards. Regulatory T cells, a specialised recently rediscovered set of T cells (explained in detail in section 3.3), also help to control immune responses and to regain homeostasis. Another important T cell homeostatic mechanism is carried out by the activated T cells themselves in a mechanism called "fratricide" (Trambas and Griffiths 2003). When T cells kill, part of the membrane from the target cell detaches and attaches to the T cell membrane. As a consequence the T cell becomes a target since now it presents target MHC peptide molecules on its surface. This mechanism allows activated T cells to become targets to other activated T cells with the same specificity, which induces T cell-T cell killing and regulates the size of the immune response. This mechanism will be further explained in chapter 4.

Even though DCs are responsible for the phenotype of the activated T cells, it is not exactly known how some T cells such as terminal effector cells are committed and programmed to kill and die afterwards, whereas other T cells are committed to gain central memory or effector memory phenotypes, which allow them to survive further. Figure 1.4 shows the transition in expression markers and functional properties of T cells from the naïve T cell status (CD27⁺ CD28⁺ CD45RA⁺ CCR7⁺ CD45RO⁻) to the central memory (CD27⁺ CD28⁺ CCR7⁺ CD45RA⁻ CD45RO⁺), effector memory (CD27⁻ CD28^{+/-} CCR7⁻ CD45RA⁻ CD45RO⁺) and terminal effector (CD27⁻ CD28⁻ CCR7⁻ CD45RA⁺ CD45RO⁻) status (Hamann, Baars et al. 1997; Sallusto, Lenig et al. 1999; Appay, Dunbar et al. 2002). CD27 and CD28 are co-activatory molecules, CD45RO and CD45RA are phenotypic markers and CCR7 is a homing chemokine receptor. Effector memory cells can become either terminal effector or central memory following a consecutive activation. Central memory cells normally remain in the lymph nodes after an infection has been cleared in order to retain immunological memory. Terminal effector cells are normally committed to kill targets and die afterwards. However it has been demonstrated that in some occasions such as in CMV infections, they can revert their phenotype to an effector memory population and live longer (Wills, Okecha et

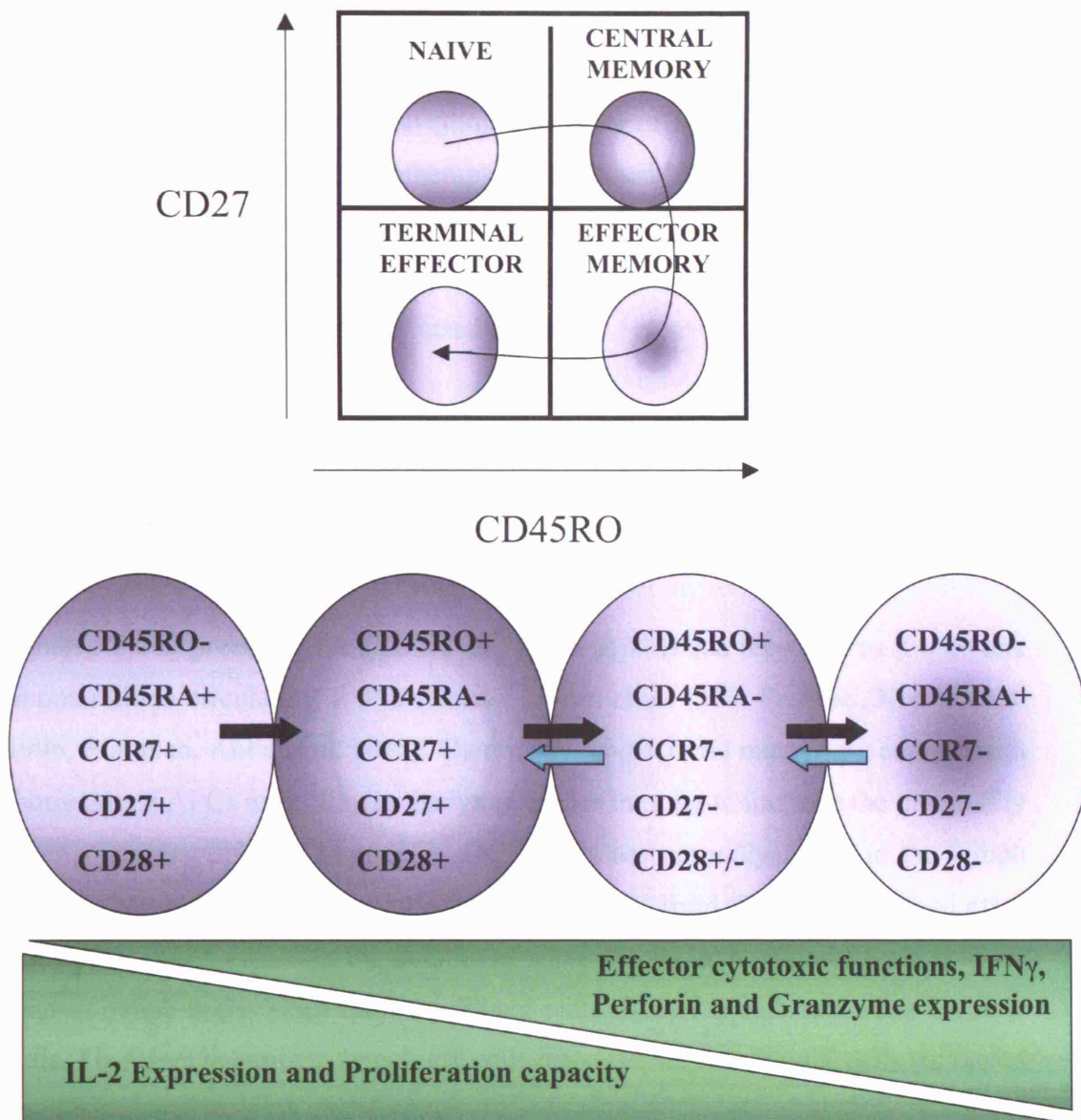


Figure 1.4.

T CELL DIFFERENTIATION: Upon activation, T cells undergo a series of phenotypical changes (naïve, central memory, effector memory and terminal effector), which determine their functional properties and anatomical distribution. Several receptors allow their identification such as CD28 and CD27, which are co-activatory molecules, CCR7, which is a lymph node homing receptor and CD45RO and CD45RA, which are phenotypic markers. The black arrows show the conventional phenotype transition from a naïve T cell to a central memory, effector memory and terminal effector. The green arrows however, show alternative directions according to what the immune system requires at certain points.

al. 2002). In summary CCR7⁺ cells are either naïve T cells waiting to be activated in the lymph nodes or central memory cells waiting in the lymph nodes ready to mount a more rapid response upon antigen re-encounter. CCR7⁻ cells are normally effector cells in the periphery travelling to the site of aggression. This is important to highlight in terms of immunotherapy as explained later in detail since infused T cells may not have the required phenotype in order to obtain the desired response. Naïve T cells and central memory T cells reside at the secondary lymphoid tissues whereas effector cells travel to affected organs.

2.3 APC - T Cell Interaction

2.3.1 T Cell Antigen Recognition

A naïve T cell precursor specific for any given peptide can represent as low as one in one million circulating T cells (Rouse, Larsen et al. 1983; Posavad, Koelle et al. 1996; Blattman, Antia et al. 2002). Therefore a single T cell must make contact with thousands of APCs every day in the lymph nodes in order to increase the probability of encountering specific peptide. Naïve T cells are normally found in the lymph nodes. They can be found in blood when they travel from the bloodstream to other lymphoid organs. However the number of naïve T cells in the blood stream is lower than in lymph nodes since they are diluted with effector T cells and other immune cells. Therefore priming truly naïve T cells responses *ex vivo* from T cells recovered from peripheral blood is challenging.

When a naïve T cell recognises an immature DC presenting self-peptides *in vivo*, it will be inactivated to avoid autoimmunity as previously explained (Jenkins 1994). However when a naïve T cell recognises a peptide presented by a mature DC, it will be activated and an adaptive immune response begins.

2.3.2 T Cell – APC Reciprocal Modulation

It is considered that dendritic cells are instrumental in whether a T cell becomes activated or inactivated. This is usually true, however what renders, conditions and licences a dendritic cell to become either activatory or regulatory is influenced by several mechanism. These include cells from the innate immune system and subsets of both CD4⁺ and CD8⁺ T cells (Altmann and Boyton 2003). For example when T cell activation is induced, dendritic cells determine whether a CD4⁺ T cell will become cytotoxic (T_H1) or helper (T_H2). This decision made by the DC is believed to be made before APC - T cell engagement. Evidence shows that different DC population induce different type of responses. Some myeloid DCs preferentially induce cytotoxic (IFN- γ mediated) responses whereas some plasmacytoid DCs preferentially induce regulatory (IL-10 mediated) immune responses (Rissoan, Soumelis et al. 1999). More importantly, it appears that the activatory or regulatory status of DCs can be reverted or modified by cytokines and cell interactions from subsets of cells from both the innate and adaptive immune system (Ruedl, Kopf et al. 1999; Noble, Leggat et al. 2003). An example of this is seen with CD8⁺ T cells that have been shown to influence the DC decision concerning CD4 differentiation towards a helper or cytotoxic function. This subset of CD8⁺ T cells has not been totally defined. The molecules or cytokines involved remain unclear too. However it is clear that subsets of CD8⁺ and CD4⁺ T cells play an important role in mediating DC commitment, which will determine the critical signal delivery following engagement, and the final phenotypic and functional antigen specific T cell properties.

2.3.3 T Cell Activation

2.3.3.1 Immunological Synapse

DCs have the ability to initiate, regulate and halt antigen specific immune responses due to a very detailed and synchronised biochemical language with T cells.

Recognition of the MHC/peptide complex (the primary signal) by its respective T Cell Receptor (TCR), can lead to either activation or inactivation of the T cell in question. The interaction between DC and T cell requires more than the primary signal and it is the specific combination of secondary costimulatory signals that are responsible for the full activation or inactivation that follows MHC-peptide/TCR engagement. This complex biochemical language takes place in the immunological synapse, which we are now starting to completely understand (fig 1.5). The immunological synapse is the superstructure of cell-cell contact communication. Adhesion molecules and cytokines also participate in this language, which also have a critical roll during DC-T Cell communication. The immunological synapse formed by lipid bilayers from both cells could last from minutes to hours to days. APCs possess thousands of different MHC/peptide complexes distributed all over their surface. Among these, hundreds of them have the exact same antigen specificity. In order to prime, activate and expand a T cell response, the APC must concentrate these MHC/peptide complexes and costimulatory molecules distributed all over its surface (a phenomenon known as capping), in the place where the immunological synapse is taking place to provide sufficient density of primary and secondary signals (Grakoui, Bromley et al. 1999). Therefore the amount of each signal is as important as the overall number of signals required for full T cell activation. APCs can translocate MHC and costimulatory molecules from all over their surface to one particular point due to their lipid bilayers, which give incredible flexibility to the synapse system. Costimulatory molecules have a critical role during T cell activation since they not only determine the quality and speed of the activation, but also generate the phenotype (i.e. effector, helper, regulatory or memory), survival potential and homing patterns of the stimulated T cells.

Listed below is a summary of what we know about the most important adhesion molecules, costimulatory molecules and cytokines. The T cell surface is the best known and best characterised cell surface in the body (Evans, Hene et al. 2003). Comprehensive transcriptome studies performed by this group reveal than the TCR, CD2, LFA-1, CD8 and CD45 receptors and co-receptors are found among the 20% most highly expressed transcripts for cell surface molecules, and that the

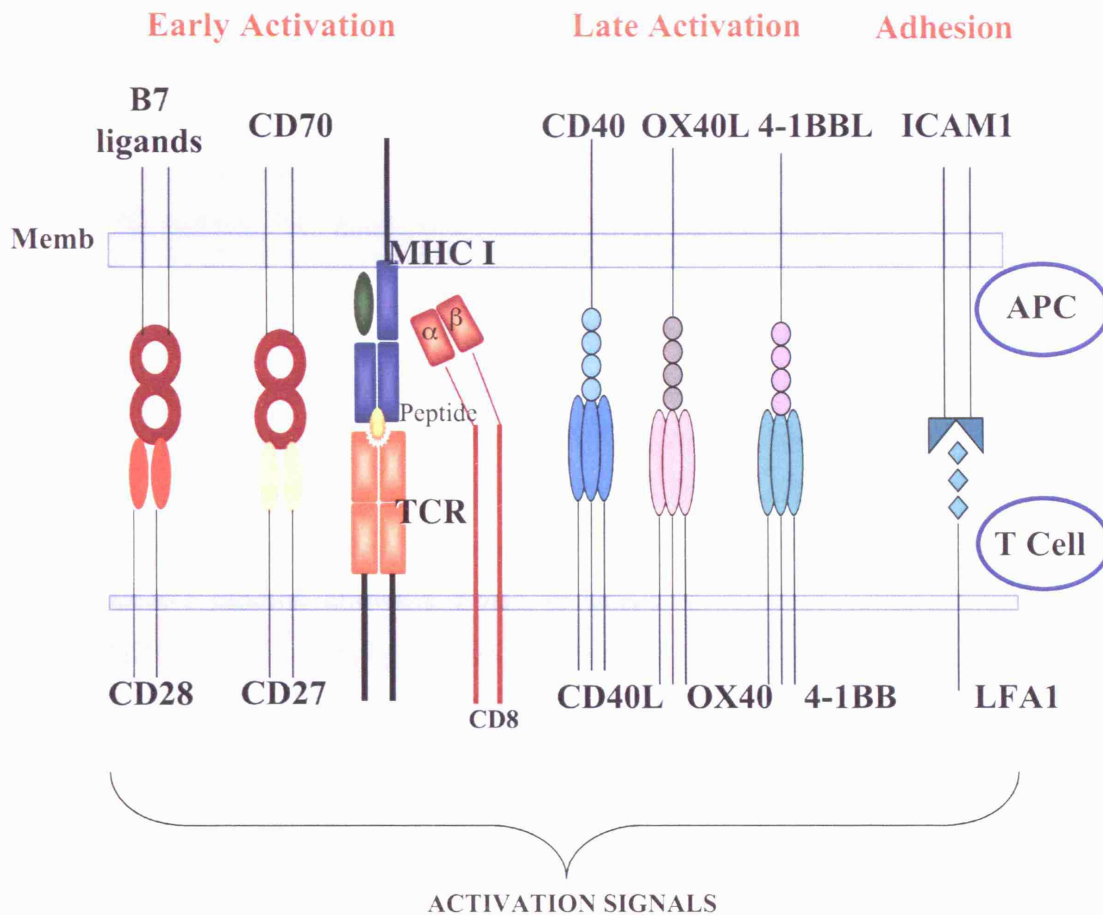


Figure 1.5.

T CELL ACTIVATION: Adhesion, early, late and survival signals are required to accomplish full T cell activation. Depending on these, the T cell phenotype, function, anatomical distribution and final destination is programmed. The immunological synapse represented here between APCs and T cells is the super-structure where this complex biochemical interaction takes place.

composition of the key surface molecules constituting the triggering apparatus of CD8⁺ T cells is largely defined.

a) Costimulatory Signals

1) Adhesion Molecules

Intra Cellular Adhesion Molecule-1 or ICAM-1, also known as CD54, is expressed on the APC surface. Its ligand on T cells is the Lymphocyte Function-associated Antigen-1 or LFA-1 (CD11a) receptor. ICAM-1 is one of the first described adhesion molecules and possesses an extremely potent synergistic effect on T cell activation when co-expressed with the early activation CD28 molecule (Wulfig and Davis 1998; Sedwick, Morgan et al. 1999). LFA-1 is thought to be the most important adhesion molecule for T cell activation since antibodies that block it inhibit the activation of naïve and effector T cells. All APCs express ICAM-1. ICAM-1/LFA-1 binding allows a T cell to screen an APC in order to find its specific MHC peptide complex. When this happens, a conformational change in LFA-1 through the TCR receptor greatly increases the strength of the immunological synapse. This allows the delivery of secondary specific signals in order to initiate the adaptive immune response. DC-SIGN, another adhesion molecule that binds to ICAM-3 (CD50) on the T cell surface, is only expressed on DCs. DC-SIGN has taken particular importance since it is both an antigen receptor recognizing pathogens through carbohydrate structures, and an adhesion molecule taking part during the immunological synapse formation. This dual function has a disadvantage (Geijtenbeek, Engering et al. 2002) as DC-SIGN is one of the target receptors for HIV infection. Due to that HIV not only produces immunosuppression by DC infection but also allows virus dissemination and spreading to T cells since it takes part in the APC-T cell immunological synapse formation (Engering, Geijtenbeek et al. 2002). Other adhesion molecules include ICAM-2 (CD102), which also binds to LFA-1 and CD2, which binds to LFA-3 (CD58). The LFA-3/CD2 interaction has also been shown to add significant potency to the ICAM-1/CD28 combination (Davis, Ikemizu et al. 2003).

2) Early Activation Signals

CD28 is expressed on the T cell surface. Its ligands are B7.1 and B7.2 (also known as CD80 and CD86), which are expressed on the APC surface. This interaction is probably one of the best studied and characterised costimulatory pathways. CD28 is essential for enhanced priming accuracy and also induces short term T cell survival and triggers T cells to secrete IL-2 without CD4 T help. One of the most important effects is that CD28 promotes Th1 cells to a cytotoxic phenotype. B7.1 and B7.2 are also the ligands for the antagonist receptor CTLA-4 (CD152), which normally inhibits proliferation and controls the magnitude of an immune response. However this can induce T cell anergy and promote regulatory T cell differentiation (Bachmann, Kohler et al. 1999; Collins, Brodie et al. 2002). Artificial APC systems that target CD28 with specific monoclonal antibodies or Fab regions do not react with CTLA-4 and the suppressive effects are avoided. Fig 1.6 shows the binding properties of the B7 ligands for the CD28 and CTLA-4 receptors. Both B7.1 and B7.2 are able to bind either CD28 or CTLA-4 and both B7 ligands possess binding biased towards CTLA-4 (Collins, Brodie et al. 2002). Despite this, T cell inhibition is not preferentially induced as these receptors and ligands are not all constitutively expressed. B7.2 and CD28 are constitutively expressed on naïve and resting cells. Therefore during antigen presentation T cell activation is favoured (fig 1.7). It is following activation that CTLA-4 and B7.1 are expressed inducing the antagonistic effect on T cells since both B7 ligands possess biased ligation towards CTLA-4 (fig 1.7). It seems now clear that evolution drove these receptors to be sequentially expressed. In summary, B7.1 is predominantly but not exclusively inhibitory whereas B7.2 is more effective with CD28 inducing activation at the beginning of an immune response. These hierarchical receptor differences influence T cell modulation (Davis, Ikemizu et al. 2003). Animal studies have shown that when B7.2 is blocked, T cell activation is severely compromised. In contrast when B7.1 is blocked, T cell inhibition is compromised and uncontrolled immune responses including autoimmunity are observed.

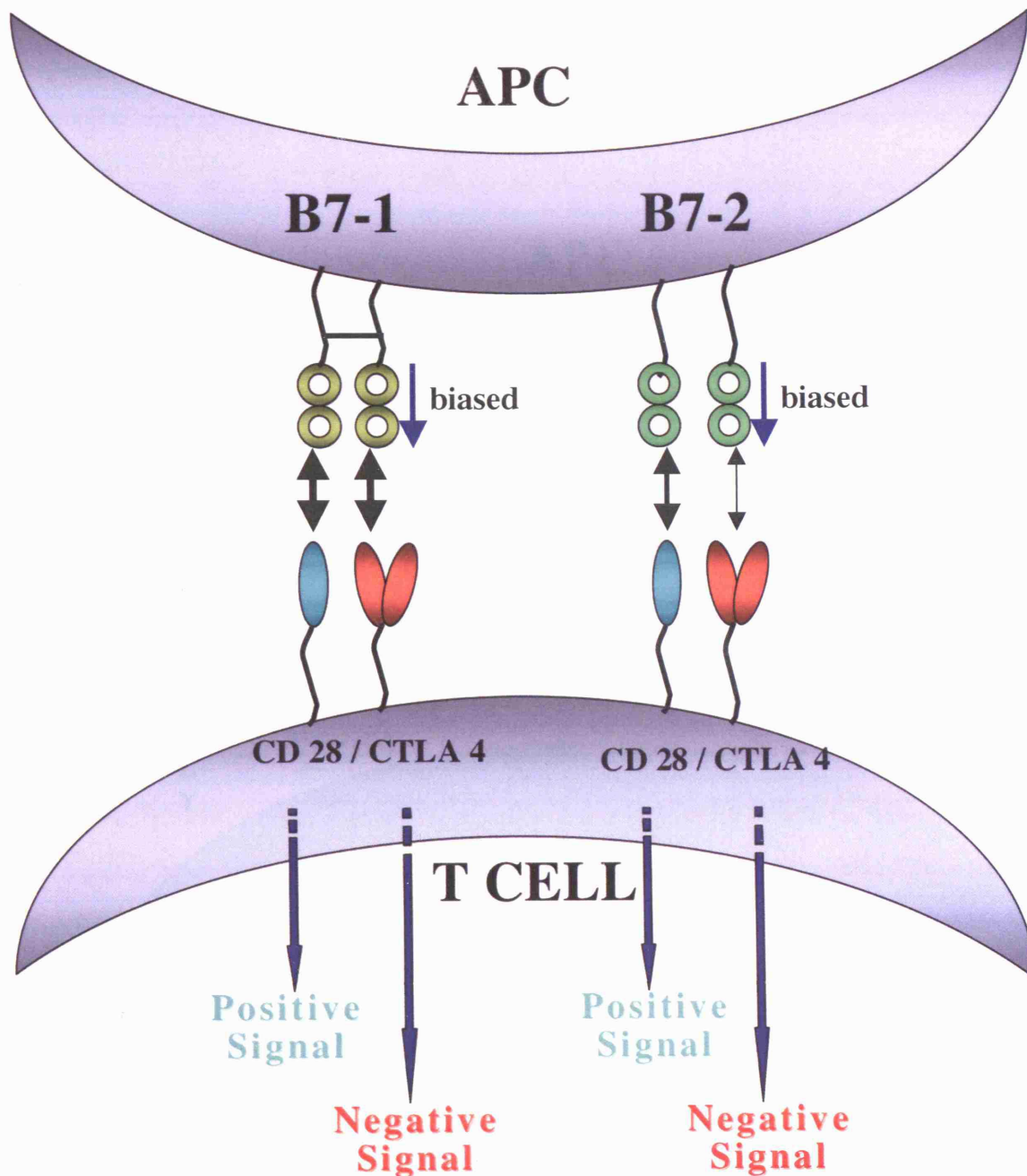


Figure 1.6.

B7 LIGANDS: B7.1, which can form dimers and B7.2, which is unlikely to form dimers, are both able to bind CD28 receptors, which are monovalent and CTLA-4 receptors, which are bivalent. However the strength of ligation is different as shown by the black double arrows. Ligation of B7 ligands to CD28 induces T cell activation, whereas B7 ligands ligation to CTLA-4 induces T cell inactivation, anergy and even programmed cell death. Both B7 ligands have biased ligation toward CTLA-4. However T cell inactivation is not preferentially induced since the receptors are not simultaneously expressed at all times.

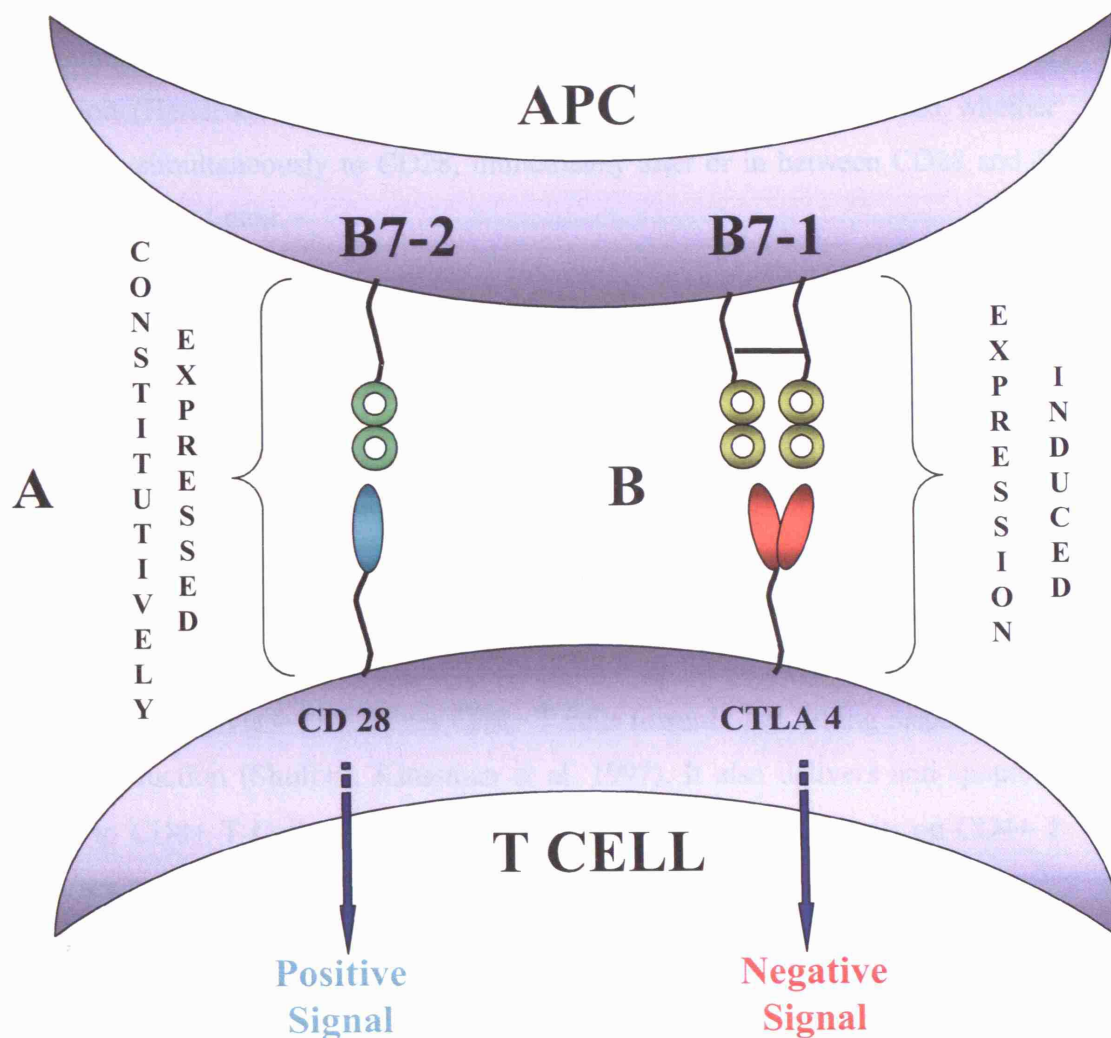


Figure 1.7.

B7 LIGANDS: B7.2 and CD28 are constitutively expressed on the surface of the APCs and resting T cells respectively. Therefore T cell activation is favored at the beginning of the immune response (A). Following activation however, B7.1 and CTLA-4 are expressed at the surface and T cell inactivation takes place since the B7 ligands possess biased ligation towards CTLA-4 (B).

Another important early activation signal is CD27. CD27 is constitutively expressed on the surface of both naïve CD4⁺ and CD8⁺ T cells. Its receptor is the CD27 ligand or CD70. Ligation is required within the first 2 days of activation. Upon engagement, CD27 strongly primes and costimulates T cells inducing clonal expansion (Hendriks, Xiao et al. 2003). It has not yet been determined whether CD27 acts simultaneously to CD28, immediately after or in between CD28 and 4-1BB as explained next.

3) Late Activation And Survival Signals

4-1BB (also known as CD137) is expressed mainly on the surface of CD8⁺ T cells. Its receptor is the 4-1BB ligand expressed on the APC surface. In contrast to CD28 and CD27, 4-1BB is not constitutively expressed on resting T cells. Its expression is induced within the first 24 hr following activation and it seems that its presence is required during the 2-6 day period of both the primary and secondary immune response following the CD28 signal (Kim, Kim et al. 1998; Bertram, Dawicki et al. 2004). Signalling via 4-1BB drives CD8⁺ T cells towards Tc1 killing responses and IFN- γ production (Shuford, Klussman et al. 1997). It also delivers anti-apoptotic signals to CD8⁺ T Cells. 4-1BB acts on CD8⁺ T cells as OX40 acts on CD4⁺ T Cells (see next).

OX40 (also known as CD134) is expressed mainly on the surface of CD4⁺ T cells. Its receptor is the OX40 ligand on the surface of the APCs. OX40 is not constitutively expressed on resting T cells but its expression is induced within the first 24 hr following activation. It is required for full CD4 priming and is also required during the 2-6 day period of both the primary and secondary immune response by delivering anti-apoptotic signals (Flynn, Toellner et al. 1998; Gramaglia, Weinberg et al. 1998). As previously mentioned, OX40 acts on CD4⁺ T cells in the same manner that 4-1BB acts on CD8⁺ T cells (Croft 2003).

CD4 cells seem to be required to accomplish T cell priming by APCs. This so called "CD4 help" is critical for CD8⁺ cells. CD40L is one of the key players in this CD4 help interaction. CD40L is expressed on CD4 T helper cells. Its ligand is the CD40 receptor, which is expressed on APCs and B cells. The effects of this receptor-

ligand interaction are bidirectional. CD40 on the APC activates a CD4 cell that has been recruited to the immunological synapse. This activated CD4 cell also activates the APC in return and the APC expresses higher levels of co-activatory signals such as CD28. This bidirectional action further activates the original CD8 T cell and priming is then accomplished successfully (Salio, Dulphy et al. 2003).

4) Expansion Signals

CD3, a co-receptor found on the surface of both CD4 and CD8 T cells, is necessary for the expression of TCR at the cell surface. It is required for signal transduction upon TCR engagement. CD3 is probably one of the most exploited signals to accomplish T cell expansion. Following its engagement with antibodies against CD3, T cell proliferation is induced.

5) Cytokines

Several cytokines are required to accomplish T cell activation and expansion, whereas others are induced upon activation. Interleukin-2 (IL-2) stimulates T, B and NK cells. It is produced by T cells upon MHC/peptide and CD28 activation. IL-2 drives cell division. The activated cells can divide two or three times a day for several days and IL-2 also induces effector phenotype differentiation. IL-15 functions in a cell-cell contact fashion between T and APC cells. It is required to prime immune responses, generate high avidity T cells and induce memory T cell phenotype differentiation. Blocking IL-15 abrogates the memory T cell pool (Klebanoff, Finkelstein et al. 2004; Alpdogan, Eng et al. 2005). IL-7 stimulates antigen specific T cell clones (Rathmell, Farkash et al. 2001) and IL-12 provides CD4 help. CD8 effector T cells and CD4 T_H1 release IFN- γ , which greatly up regulates MHC class I and II expression on APCs and infected cells, activates macrophages and blocks viral replication. It also induces elimination of viruses from infected cells with or without killing the infected cells. Both CD8 T cells and CD4 T_H1 cells produce TNF- α and β , which can be cytotoxic by production of

nitric oxide (a powerful cytotoxic agent). However TNF- α and TNF- β can also induce programmed cell death by binding to the TNFR-1 receptor. CD4 T_H2 cells secrete IL-4 and IL-5, which activate B cells and eosinophils respectively, and IL-10 and TGF- β , which inhibit T cells and APCs.

2.4 T Cell Mediated Killing

Once a T cell has been activated and clone expansion has occurred, CD8 and CD4 killer cells remain in the lymph nodes and gain a combination of receptors that allow them to travel to the site of infection or tumour growth as previously explained in section 2.2.2.4 and figure 1.4. CD4 killing is commonly underestimated but it is well established and documented (Yanai, Ishii et al. 2003). However most studies have focused on CD8+ mediated killing. A CD8+ T cell is capable of killing more than one target cell but kills one at the time. The T cell detaches and attaches rapidly during killing delivering the so-called “kiss of death” (Trambas and Griffiths 2003). Cytotoxic cells can kill by two main different mechanisms. The first is via the use of pre-formed cytotoxic enzymes (perforin and granzymes) entrapped in vesicles (Peters, Borst et al. 1991; Trapani and Smyth 2002), and the second mechanism is via the FAS ligand. However other T cell killing mediated mechanisms exist such as apoptosis induced by TNF α or TRAIL (TNF-related apoptosis inducing ligand). When cytotoxic enzymes are released, their specific delivery is extremely well regulated in order to avoid the damage of surrounding healthy cells and the T cells themselves. First a tight and narrow bond is created between T cell and target cell upon TCR-MHC peptide complex recognition, which also triggers a reorientation of the secretory vesicles of the T cell towards the target cell. In doing this, the cytotoxic enzymes are delivered accurately and correctly without collateral damage. Perforin produces pores in the membrane of the target cells causing the osmotic and oncotic equilibrium of the target cell to be lost and thus cell death. These pores created by perforin also allow the entrance of granzymes into the target cell (Shiver and Henkart 1991; Shiver, Su et al. 1992; Nakajima, Park et al. 1995). Granzymes are digestive enzymes such as trypsin and

chemotrypsin that activate caspases, proteases which then directly target and digest the DNA leading to apoptosis.

The Fas ligand killing mechanism is used by both CD8 and CD4 T_H1 cells. Upon engagement with target cells via the Fas receptor, Fas ligand (also called CD95L), which possesses a death domain, induces apoptosis on the target cell by activating the caspases cascade. The granzyme-perforin pathway normally dominates in anti tumour and anti viral responses (Yanai, Ishii et al. 2003), whereas the Fas ligand mechanism is usually reserved for killing the remaining self lymphocytes after an infection has been cleared although it can also be used to kill infected cells.

In addition to direct killing, T cells also secrete cytokines. For CD8+ T cells, the main cytokines produced are IFN- γ , TNF- α and TNF- β . IFN- γ blocks viral replication and can induce elimination of the virus from infected cells without killing the cells. IFN- γ can be pre-formed but it is normally synthesized *de novo* upon antigen recognition by T cells. Therefore T cell IFN- γ detection is often used to measure T cell functionality in anti viral T cell responses. IFN- γ also induces up regulation of MHC class I molecules on target cells. This effect is very important since T cells can only kill target cells that bear the specific MHC peptide complex. Some tumour or infected cells down regulated MHC expression as an evasion mechanism (section 3.1.1). IFN- γ reverts this mechanism and makes the target cells "more visible" to T cells. IFN- γ also recruits APCs to the site of infection in order to amplify the immune response if needed. CD4 T_H1 cells also produce IFN- γ and activate macrophages to induce killing of their intracellular pathogens. As previously mentioned, TNF- α and TNF- β can be cytotoxic themselves by production of nitric oxide. However TNF- α and TNF- β can also induce programmed cell death by binding to the TNFR-1 receptor. Programmed cell death or apoptosis produces self-cell death from within and even though it can take hours for a cell to die, apoptosis is effectively induce in the first 5 minutes of T cell-target cell contact. A hallmark of apoptosis is the fragmentation of DNA into 200 base pair pieces since endogenous endonucleases are activated to cut the DNA between nucleosomes. These enzymes are also capable of digesting viral DNA. Therefore

the Fas ligand killing mechanism is sometimes used by T cells to control viral infections and avoid pathogen spreading.

Whatever killing mechanism a T cell is committed to use, part of the target cell membrane detaches during T cell-target engagement and becomes part of the T cell membrane. This process makes any active killer T cell a target since T cells themselves "present" MHC target molecules and become now vulnerable targets from T cell killing in a mechanism called "fratricide" (Trambas and Griffiths 2003). This T cell killing mechanism forms part of the natural way the immune system controls immune responses and avoids excessive T cell proliferation. T cell homeostasis is then restored as previously explained in section 2.2.2.4.

2.4.1 T Cell Affinity, Avidity And Recognition Efficiency

For a T cell to kill safely, it needs to recognise a target cell in an accurate antigen specific fashion. Binding of a T cell to its target is ultimately achieved by a sequence of events. The T cell receptor structure itself determines affinity for its ligand. Affinity it is intrinsically given to T cells previous target encounter, nevertheless T cell affinity commits the T cell to a certain target. In order to induce killing, a chain of events during target recognition produces what is known as the T cell-target avidity. Avidity is therefore given by a combination of the T cell affinity for a given MHC peptide complex, the strength of the CD8-MHC co-receptor interaction, the localization of TCR dimers during recognition, the recruitment of signalling and activation molecules to the TCR-CD3 complex and the contribution of costimulatory molecules during the immunological synapse (Kedzierska, La Gruta et al. 2005). Two T cells with different affinities (TCRs) for the same peptide will translate into two different overall avidities when targets are recognised. An example of this is sometimes observed in tetramer staining plots as shown later in chapter 4, figure 4.2. When poly or oligo clonal responses against the same peptide are induced, two (or more) separated tetramer positive specific populations can be observed since the TCR affinities vary in the T cell pool. Even though both populations are peptide specific, they are detected slightly differently by Flow

cytometry since the affinity of the TCRs and the overall avidities for the same target may be different.

Classically, avidity has been used and referred as the binding of a multivalent molecule to its target, such as the binding between an antibody and its target, and not between two cells. Therefore to “avoid” confusion, the term recognition efficiency was introduced to describe the overall binding between a T cell and its target (Rubio, Stuge et al. 2003; Kohrt, Shu et al. 2005), which reflects all the cumulative effects previously described, which eventually lead to target killing.

2 IMMUNOTHERAPY

The immune system comprises a complex army of cells that communicate in an extremely well-balanced manner. Since Edward Jenner developed the successful small pox vaccine using crude extracts of a less virulent virus in the 1790s, researchers have tried to accomplish protection against many other diseases including cancer. Vaccination with crude tumour extracts did not accomplish protection, but the idea of immunotherapy began and currently the developments and strategies against diseases are revolutionising the way we live and how long we live.

The manipulation of different branches of the immune system to achieve immunity continues and varies from the use of monoclonal antibodies and cytokines to specific subsets of cells. It has been only 30 years since scientists first developed synthetic antibodies. However now antibodies such as HERCEPTIN, used to treat breast cancer, and CAMPATH, used to deplete T cells in bone marrow transplantation, are finally becoming standard therapeutic approaches.

The idea of targeting the immune system against cancer began when doctors noticed that some tumours were eliminated when they were infected with pathogens such as viruses. That suggested that tumours are not normally immunogenic and evade recognition by the immune system. However when “danger signals” provided by pathogens are present, the immune system is able to recognise the tumours and may eliminate them entirely. Treatment of bladder cancer with BCG vaccine is an

example. When the BCG vaccine, normally used to induce resistance to tuberculosis, is injected into the bladder, it causes inflammation and the release of danger signals, which activate the immune system and lead to tumour shrinkage (Alexandroff, Jackson et al. 1999).

Other immunotherapeutic approaches include the use of cytokines such as IFN- α , which is delivered systemically to treat different types of cancer such as leukaemias. IFN- α can affect tumour cells both directly and indirectly through up regulation of MHC peptide molecules.

Antibody-based therapies can stop tumours by such mechanisms as attaching themselves to tumour cells and alerting immune cells to the tumour. Tumour specific antibodies can carry drugs, radioactive particles and enzymes. These observations have confirmed that the immune system plays a definitive role in the eradication of malignant diseases. More recently however, the use of subsets of cells such as CD8⁺ or CD4⁺ T cells are making major impact in viral and cancer immunotherapy (Falkenburg, Wafelman et al. 1999; Oelke, Krueger et al. 2005) as explained below.

2.1 T Cell Mediated Immunotherapy

Scientists have developed a vast range of therapeutic tools to fight cancer, viral infections and degenerative diseases. Chemotherapy, radiotherapy, surgery, organ and tissue replacement, hormones, drugs and neurotransmitters are some examples of this. However utilizing the immune system to fight tumours and viruses mimics a natural phenomenon. As previously explained, immunotherapy consists on the usage of cytokines, antibodies, serum factors, proteins, peptides and cells in order to augment direct and indirect effects in the overall immune *in vivo* response. T cell mediated immunotherapy is a subdivision that uses one of the most pivotal cytotoxic cells of the adaptive immune system in order to deliver a potent effect. CD8⁺ and CD4⁺ T cells are capable of killing tumour cells, viral infected cells and can rescue and cure patients with cancer as explained later in detail in chapters 4 and 5. In some cases the immune system needs to be helped, supported or boosted

in order to accomplish eradication of the disease. Active and adoptive immunotherapy are two powerful mechanisms of accomplishing this (detailed in sections 2.1.1 and 2.1.2) In order to produce a T cell mediated immunotherapeutic approach against a particular tumour, it has to be carefully assessed whether the target tumour fulfils some prerequisites such as the expression of MHC peptide complexes, and whether target peptides presented by these MHC molecules can be eventually targeted by specific T cells following antigen presentation (Pawelec, Engel et al. 1999). There are several antigens presented specifically by tumours and viral infected cells that can be targeted by T cells such as BCR-ABL in chronic myeloid leukaemia (Clark, Dodi et al. 2001), Wilm's tumour antigen expressed in Wilm's tumour and leukaemias (Gao, Xue et al. 2003), telomerase (Vonderheide, Hahn et al. 1999), expressed in most tumours and the pp65 immunodominant peptide in cytomegalovirus infections among many others. However T cell mediated immunotherapy was surrounded by pessimism for some time (Rosenberg, Yang et al. 1998; Rosenberg, Yang et al. 2004). In their review of 2004, Rosenberg et al discuss how tetramers, a sensitive technique used to identify antigen specific T cell populations, have been used to detect and demonstrate the *in vivo* generation and/or survival of antigen specific T cells in patients undergoing immunotherapy trials. These results created optimism in the field and over-expectation since the strategies appeared to work. Researches then realised that the generation of antigen specific T cells as detected by tetramers did not necessarily correlate with a clinical response. Therefore both adoptive and active immunotherapy needs detailed analysis as a new field has evolved involving the quality of the T cells generated, their survival, trafficking to the relevant areas, behaviour in the tumour microenvironment and functionality, which will ultimately determine whether the tetramer positive cells will have an effect on the patient's clinical outcome. An underestimated feature is that time plays a critical role during immune responses. An effective anti tumour T cell response might be taking place but the time that it needs to eliminate the target may not be sufficient. Therefore all these factors must be considered carefully before taking a decision about the use of T cell mediated immunotherapy.

2.1.1 T Cell Adoptive Immunotherapy

T cell adoptive therapy is currently in clinical trials. The protective properties of transferred CD8⁺ T cells has been assessed in several studies: Passive administration of T cells from antigen experienced animal donors to naïve animal recipients protects the naïve animals from tumour challenge, whereas elimination of endogenous CD8⁺ T cells abrogates both protective and therapeutic anti tumour effects. Furthermore, extensive T cell infiltrates are commonly found in tumours and allografts undergoing rejection (Schriber 2003). Even though CD8⁺ T cells require CD4 help and interaction with antigen presenting cells, the CD8⁺ T cells are the final effectors in most of the models. Therefore, the *ex vivo* activation and expansion of T cells is of great interest. The advantages and disadvantages of this process will be discussed in detail in chapter 4. However one of the main advantages of adoptive immunotherapy is the ability to select highly activated cells in greater numbers in order to deliver a clinical effect. One of the main drawbacks however is that these cell allografts can cross-react with the patient producing potentially lethal responses. It has also been observed that *ex vivo* manipulated cells can develop a compromised *in vivo* survival after infusion (Dudley and Rosenberg 2003). Nevertheless despite these drawbacks, adoptive T cell immunotherapy has demonstrated curative effects in humans in malignant diseases such as chronic myeloid leukaemia (Falkenburg, Wafelman et al. 1999; Dazzi, Szydlo et al. 2000), large vascularized melanomas refractory to treatment (Dudley, Wunderlich et al. 2002) and otherwise fatal viral infections such as cytomegalovirus reactivation in patients undergoing bone marrow transplantation (Peggs and Mackinnon 2004; Peggs and Mackinnon 2004). Therefore T cell adoptive immunotherapy promises a lot for the future as an immunotherapeutic strategy.

2.1.2 T Cell Active Immunotherapy

T Cell Active Immunotherapy refers to the generation of T cells *in vivo* and the generation of endogenous targeted responses towards specific antigens. There are

many different approaches that have been explored in order to accomplish this. These vaccines include the use of natural peptides (Pinilla-Ibarz, Cathcart et al. 2000), synthetic modified and enhanced peptides also called heteroclitic peptides (Monsurro, Nielsen et al. 2001), naked DNA (Gurunathan, Klinman et al. 2000), peptide pulsed dendritic cells (Nestle, Alijagic et al. 1998; Brossart, Wirths et al. 2000) and recombinant viruses encoding tumour antigens (Van Pel, De Plaen et al. 2001) among others. All of these strategies have advantages and disadvantages as described in chapter 5. However the rate of failure or success is mainly determined by factors such as the inoculation route, presence of danger signals, delivery and presence of co-activatory signals, the time of *ex vivo* manipulation for the case of peptide pulsed DCs, and dose. Active immunotherapy possesses the main advantage of generating own endogenous cells with no or very little cross-reactive capabilities and the generation of a more physiological response. However it also possesses disadvantages such as tolerance induction rather than immunogenic responses if the appropriate conditions are not delivered correctly. There is also a potential scenario when the intended targeted T cells to be activated and expanded have already been deleted, killed or anergised by the tumour and pathogen immune evasion and suppression mechanisms, which will be explained below. Nevertheless active immunotherapy represents a great therapeutic tool since the self immune system is used to mount effective immune responses without T cell manipulation and the risk of concomitant unwanted responses by donor infused cells.

3 TUMOR AND PATHOGEN ESCAPE

Tumours and pathogens possess a range of immune evasion and suppression strategies. Pathogen strategies are the result of years of evolution, whereas tumour strategies are mainly the result of random somatic mutations that result in a progressive malignant transformation. Some strategies suppress or prevent immune responses while others are simply directed to escape recognition.

3.1 Immune Evasion Strategies

In order to avoid recognition, tumour and infected cells can either down regulate the receptors required by the immune system to elicit cytotoxicity or simply delete them completely. In doing this they protect themselves from attack.

3.1.1 Class I And Class II Down regulation

Without peptide presentation by antigen presenting and target cells, T cell activation, recognition and cytotoxicity cannot occur. Many viruses for instance, have evolved evasion strategies that directly target antigen presentation by both DCs and infected cells. As previously described in section 2.1.3, several proteins in the cytosol and the endoplasmic reticulum (ER) are critical for MHC-peptide binding, loading and transportation to the cell surface. When Herpes viruses such as CMV infect a dendritic cell, inhibition of TAP-1 and TAP-2 proteins can occur, and as a consequence peptide transport from the cytosol to the ER is blocked. Therefore MHC peptide binding is obstructed and viral presentation to T cells is prevented. CMV also promotes degradation of MHC molecules further preventing MHC expression at the cell surface. Other viruses such as adenoviruses retain MHC peptide molecules in the ER with the same final outcome. Tumour cells can be deficient in TAP proteins (Johnsen, Templeton et al. 1999). All these mechanisms produce an overall down regulation of MHC peptide complexes on the surface of the APCs and infected cells. When APCs are targeted, MHC down regulation translates in failure of antigen presentation. When other cells are targeted, MHC deregulation leads to escape from T cell mediated killing.

3.1.2 Class I and Class II Total Loss

Tumour cells may delete the β_2m or part of the HLA DNA sequence as a consequence of the accelerated rate of somatic mutations that they undergo. The outcome of such deletions translates into partial or total loss of MHC class I and/or

II expression at the cell surface, making the cell practically undetectable and resistant to cytotoxic T cells. However this lack of MHC molecules should induce NK cell mediated lysis (Moretta, Mingari et al. 1996). Therefore tumour cells that survive or relapse are resistant to both T cell and NK cell killing. Therefore tumours must display a balance between MHC down regulation and allelic loss in order to evade immune responses.

3.2 Immune Suppression Strategies

The tumour microenvironment is rich in immunosuppressant factors including cytokines, cells, negative costimulatory receptors and enzymes. These strategies are directed towards immune cells in order to render them inactive.

3.2.1 Immunosuppressive Receptors And Cytokines

Leukaemic (Kamihira, Yamada et al. 1997) and colon carcinoma cells (Okada, Komuta et al. 2000) for instance, have been shown to express the Fas ligand cell-death signal receptor, which induces T cell death upon engagement. Other tumours such as melanoma and coetaneous carcinomas secrete immunosuppressive cytokines such as TGF- β (Moretti, Pinzi et al. 1997) and IL-10 (Kim, Modlin et al. 1995), which produce immune cell unresponsiveness and disease progression.

3.3 Immune Tolerance Mechanisms

MHC/peptide complex presentation to naïve T cells in the absence of co-activatory signals by immature APCs induces deletion and tolerance to the particular peptide presented (Jenkins 1994). Since tumours are derived from self-cells and express self-antigens, they do not normally produce danger signals and are poorly immunogenic, not allowing APCs to mature upon encounter with released tumour antigens and tolerance is sometimes therefore promoted.

One of the most interesting tolerogenic mechanisms has been the rediscovery of regulatory T cells (T_{regs}). T_{regs} produce immunosuppressive cytokines and co-inhibitory signals, and protect some cell types from killing. They are often detected at the tumour site (Liyanage, Moore et al. 2002) and in circulation of cancer patients (Wolf, Wolf et al. 2003). There are several types of T_{regs} . $CD4^+ CD25^{\text{HI}} \text{Foxp3}^+$ T_{regs} are natural occurring T cells selected in the thymus during positive and negative selection (Sakaguchi 2004). However other T_{regs} are generated in the lymph nodes by regulatory and/or immature DCs (Mahnke, Knop et al. 2003) that lack expression of co-activatory signals or that have been targeted by immunosuppressive cytokines produced by tumours. Other T_{regs} are generated *in situ* at the tumour site by immunosuppressive cytokines produced by the tumour itself or by other types of T_{regs} present at the site. The presence of T_{regs} in cancer patients is believed to be one of the major factors for disease relapse and disease progression since their removal in animal models has been shown to restore antigen specific T cell immunity and rejection (Golgher, Jones et al. 2002; Tanaka, Tanaka et al. 2002). T_{regs} have retaken particular interest since they play major roles in immunoregulation. T_{regs} in general suppress immune responses, protect the body from self attack and have been shown to protect allogeneic transplants from rejection (Taams, Vukmanovic-Stejic et al. 2003). However tumours and pathogens have evolved protection mechanisms that induce peripheral T_{regs} , which will protect them. Other type of T_{regs} include Th3 T cells, which produce TGF- β and Tr1 T cells, which produce IL-10, both immunosuppressive factors. These latter two subtypes are $CD4^+$ T cells, not necessarily $CD25^{\text{HI}}$, but Foxp3^+ , a suppressor transcription factor discovered by Sakaguchi in 2003 and long considered the hallmark of natural occurring T_{regs} (Sakaguchi 2003). It is believed that these cells do eventually gain the $CD25^{\text{HI}}$ phenotype. Natural occurring $CD4^+ CD25^{\text{HI}} \text{Foxp3}^+$ T_{regs} are generated by antigen presentation in the thymus through TRC recognition. Peripheral T_{regs} however can be rendered regulatory by several mechanisms. It is still controversial whether $T_{\text{reg}} - T$ cell (which will become a T_{reg}) contact is required or whether secretions of immunosuppressive cytokines in selected microenvironments are sufficient to induce regulatory functions. However since T_{regs} express the co-

inhibitory CTLA-4 signal, a cell-cell interaction is likely. However it is still unknown whether this cell-cell interaction is antigen specific as when T_{regs} inhibit IL-2 production and generate IL-10, IL-4 and TGF- β , they arrest CD4 and CD8 T cells for several antigen specificities (Thornton and Shevach 1998) even when fully activated by matured APCs (Oldenhove, de Heusch et al. 2003). Regulatory CD8⁺ Foxp3⁺ T cells have also been recently described and are also thought to play major roles in immunosuppression. Interestingly peripheral regulatory and/or tolerogenic state can be reverted by the use of activatory or fully activated APCs (Takahashi, Kuniyasu et al. 1998). Therefore achieving a balance and fine equilibrium between activated and regulatory T cells is a major breakthrough since the manipulation of this balance holds the key for transplant tolerance, autoimmunity and tumour rejection.

4 NEW THERAPEUTIC TECHNOLOGIES

4.1 Liposome Technology

Liposomes are spherical vesicles formed from bilipid membranes with liquid interiors that can be manufactured to a range of nano sizes and dependent on the purpose can be generated from different lipid combinations. Liposomes are very stable in culture and *in vivo* (Vingerhoeds, Storm et al. 1994; Iden and Allen 2001; Maruyama 2002). Since liposomes possess bilipid membranes, they can form immunological synapses with cells mimicking natural cell interactions (Prakken, Wauben et al. 2000; Giannoni, Barnett et al. 2005). They have therefore served as one of the main models for biological membranes and have been approved for human use (James 1995). Liposomes used for therapeutic purposes avoid rapid recognition by macrophages (Iden and Allen 2001; Maruyama 2002), one of the main liposome clearance mechanisms, allowing the liposomes to circulate in the body for several days. These liposomes can also be coated with proteins, carbohydrates and other lipids. Liposomes that are targeted are termed immunoliposomes. These immunoliposomes can be generated traceable *ex vivo* and

in vivo and represent a clean system to evaluate delivery and receptor interactions. Due to their size, liposomes possess particular kinetic behaviour. Details regarding these properties will be fully explained in chapters 2 and 3.

4.2 Nanotechnology

Nanotechnology does not only concern scientific and medical research. It includes different areas and unrelated fields that have such diverse applications as “tools in miniature” for chemistry, energy production, communication and many others. Nano devices form a large set of materials with distinct optical, electrical, mechanical and magnetic properties. They have already been used to produce from everyday objects such as stain resistant fabrics, reinforced tyres and suntan lotion to high profile devices such as NASA micro robots.

Nanotechnology refers to the interactions of natural and engineered materials at a very small (nano) scale, typically in the scale of 100 nanometres or smaller. However for biomedical nanotechnology purposes, it is not just the size that matters. To be considered as such, nano devices must possess one or more clinical and research application such as ability to detect disease, provide information on location within the body, delivery of drugs, killing of malignant cells, mounting or controlling immune specific responses or tumour imaging all with the aim of generation of “smart” targeted and traceable devices.

Human cells are in the scale of 10,000 to 20,000 nm in diameter, nanoscales devices smaller than 50 nm can easily enter most cells, whereas those smaller than 20 can transit in and out the blood vessels freely. Nanotechnology is an area undergoing massive interest. Optimism is so great that some groups such as the U.S Department Of Health and Human Services and the National Cancer Institute have put themselves the goal of eliminating death and suffering from cancer by 2015 with the use of nanotechnology. However such optimism must go hand in hand with caution. Nanotechnology possesses hypothetical risks. As yet there are no environmental or health issues with the use of nanotechnology. However there is a scenario described as “grey goo”. This postulates that a nano organic self-replicating “smart” organism

engineered through nanotechnology could become a malignant substance. In other words, grey goo is a massive hypothetical environmental tumour or cancer that may become impossible to eradicate. Social risks have also been raised such as military organizations using nanotechnology to trace and/or kill human targets at long distances using invisible nano devices carrying poisonous substances. These issues are currently being discussed in order to regulate what areas researchers will be allowed to exploit. Cancer nanotechnology and immuno nanotechnology are one such area.

5 SUMMARY AND AIM: DEVELOPMENT OF A NOVEL NANOTECHNOLOGY BASED ARTIFICIAL APC FOR T CELL MEDIATED IMMUNOTHERAPY

During this introduction we have highlighted how the immune system works, its potential, its uses when manipulated for therapeutic purposes and how to overcome tumour and viral immunosuppressive strategies in order to eradicate tumours and pathogens. As mentioned, T cells are one of the most pivotal cell types in the human adaptive immune system due to their cytotoxic capabilities. They are able to eradicate primary, metastatic (Kawakami, Eliyahu et al. 1995; Naito, Saito et al. 1998), relapsed tumours (Zhang, Conejo-Garcia et al. 2003) and can ameliorate otherwise fatal viral infections. Unfortunately antigen specific T cell numbers from patients and even from donors are often small. These cells need to be expanded first in the majority of the cases [with a few specific exceptions (Cobbold, Khan et al. 2005) as explained next] in order to deliver a significant clinical impact in patients (Dudley and Rosenberg 2003). Not surprisingly therefore, the activation and expansion of T cells either antigen specific or polyclonally, has become one of the main strategies for immunotherapy and immune gene therapy. The activation and expansion of naïve T cells known as T cell priming and the expansion of memory T cells are both components of successful T cell specific treatments against tumour and viral antigens as well as antigen specific T cell reconstitution after Bone Marrow Transplantation (BMT) (Peggs and Mackinnon 2004; Peggs and

Mackinnon 2004). Antigen presenting cells (APCs) are naturally responsible for T cell activation, expansion and survival. However *in vivo*, immune evasion and suppression strategies by tumours and pathogens directed either at APCs or at T cells produce such an imbalance that immune responses are impaired. APCs play a central role since they mount and organise all adaptive immune responses. Therefore currently there are many cellular and non-cellular systems available to mimic antigen presentation for T cell activation and expansion (Kim, Latouche et al. 2004). Natural and artificial APCs are current powerful tools that translate already into clinical impact in patients. However factors such as potency, speed of expansion, quality of the T cells generated, specificity and bio safety vary among different models. Even though *ex vivo* artificial APC systems are available for accomplishing T cell expansion for further therapeutic infusion (Adoptive Immunotherapy) (Peggs and Mackinnon 2002; Peggs, Verfuether et al. 2003; Peggs and Mackinnon 2004; Oelke, Krueger et al. 2005), the main drawback of these systems is that many if not all of them rely on one critical factor: *ex vivo* manipulation. The problem with this is that the longer the *in vitro* T cell culture during the process, the shorter the *in vivo* T cell survival after infusion (Dudley and Rosenberg 2003). Therefore the dilemma to shorten as much as possible the *in vitro* manipulation and accomplish fast high T cell numbers is whether to isolate the antigen specific T cells first and expand them later or vice versa. However whatever the approach, T cell *ex vivo* manipulation is not ideal. Therefore artificial systems are required to accomplish T cell expansion *in vivo* as an active immunotherapy strategy. Recently strategies involving antigen specific T cell sorting from donors followed by immediate infusion into patients after bone marrow transplant (Cobbold, Khan et al. 2005), seem to "sacrifice" the final T cell number for *in vivo* survival post infusion by shortening the *in vitro* T cell culture to 4 hours. This strategy seems to work after BMT partially because there is a "privilege empty environment" following extensive chemotherapy and radiotherapy, which allows infused T cells to replicate freely. This phenomenon is not likely to occur in other patient groups. Despite the short culture T cell sorting is feasible for treating CMV reactivation following BMT, however when it comes to tumours no healthy donors

are available to provide a source of T cells. *In vivo* artificial APCs would be therefore ideal for active cancer immunotherapy.

To manipulate T cells in patients, different scenarios must be identified in order to know whether the patient is naïve or otherwise for a particular antigen. Some patients may have an established tumour or infection simply because the immune system was unable to identify the target in which case the patient remains naïve for the tumour antigen. The immune system of other patients might have identified the target at a certain point, but were unable to eradicate it. These patients may have primed specific T cell clones. Some patients may possess functional specific high avidity clone(s) against the target whereas in some others, the specific T cell clone(s) might have been already anergised or even deleted from the repertoire. This is important to highlight since the kinetics of primary and secondary adaptive immune responses are fundamentally different. Therefore each type of patient requires a particular type of T cell manipulation. Also important to remember is that whatever kinetics a T cell follows, whatever role it plays and without even considering how beneficial or detrimental it can be, its activation or inactivation relies on one critical factor: “Antigen Presentation”. This critical event is responsible for the final fate and destination of every single T cell clone (from activation to function, behaviour and even programmed cell death). Therefore the key to T cell manipulation is the complete and perfect understanding of the interaction between T cells and antigen presenting cells. Details concerning the biochemical communication between T cells and APCs taking place in the immunological synapse are starting to be revealed. So far it is known that *ex vivo* it could be simultaneous expression of CD28, CD27, 4-1BB and OX40 on T cells. However it is not known what the exact kinetic differences are, whether they all are equally functional, equally relevant and whether they induce cell proliferation or just suppress cell death. Having said this, the model seems to work as shown in figure 1.5 (Shuford, Klussman et al. 1997; Flynn, Toellner et al. 1998; Gramaglia, Weinberg et al. 1998; Kim, Kim et al. 1998; Wulfiging and Davis 1998; Sedwick, Morgan et al. 1999): CD28 and CD27, constitutively expressed on naïve T cells, are essentially required for both CD4 and CD8 full priming and expansion (Hendriks,

Xiao et al. 2003). In an overlapping fashion OX40 takes over the CD4⁺ T cell response, whereas 4-1BB takes over the CD8⁺ T cell response (Croft 2003; Bertram, Dawicki et al. 2004). Although the primary signal given by MHC/peptide complexes is essential, it is only required in the first 20 hrs of the initial T cell-APC antigen presentation. After this, the costimulation and the immune response continue in an antigen independent manner. Furthermore it seems that multiple stimulations via MHC/peptide complexes generate clonal exhaustion, anergy and deletion (Maile, Wang et al. 2001).

As mentioned previously, APCs are central protagonists for the immune system since they are responsible for mounting and organising adaptive immune responses. Unfortunately in certain instances, professional APCs cannot present antigens correctly. The main reason is due to a vast range of immune evasion and suppression strategies exhibited by pathogens and tumours that directly target the APCs and/or the T cells in order to achieve a survival advantage. As a consequence, immune responses against some tumours and pathogens are defective, compromised or even never generated. However despite this it is possible to generate an effective immune response when natural antigen presentation is by-passed by artificial antigen presenting systems *ex vivo* or *in vivo* (Wolfers, Lozier et al. 2001; Thery, Duban et al. 2002; Andre, Chaput et al. 2004; Herrera, Golshayan et al. 2004). Donor Lymphocyte Infusions (DLI) for example, have demonstrated that fully activated T cells from a particular donor are capable of killing tumour cells and eradicating cancer on infusion into patients (Kolb, Mittermuller et al. 1990; Drobyski, Keever et al. 1993; Mackinnon, Papadopoulos et al. 1995; Falkenburg, Wafelman et al. 1999). The significance of this and other results is that T cells themselves have the capability of both continuing the immune response and eradicating the specific target once antigen presentation in the patient has been by-passed.

Bypassing the APC maturation process in some patients and antigen presentation in others seems to be the key to effective and lasting immune responses. However it is very important to highlight that even though natural antigen presentation can be successfully by-passed by artificial systems, this does not necessarily mean that

pathogen or tumour tolerance will be broken. In other words, the fact that functional T cells can be artificially activated and expanded does not mean that these T cells will kill and eradicate the infected or tumour cells. This is because target cells can be protected by regulatory T cells, can lose or down regulate MHC-peptide complexes, mutate or change the target peptide or secrete T cell suppressive cytokines making a fully activated T cell into a completely unresponsive one. Nevertheless tumour and pathogen tolerance can be broken by by-passing or improving antigen presentation. Therefore several groups, including ours, are trying to develop models and systems to accomplish this. In order to do that, the current systems must be analysed to develop a novel superior system. It is well known for instance that *ex vivo* manipulation and full activation-maturation of DCs can overcome some of the immune evasion and suppression mechanisms. These DCs can efficiently and effectively activate and expand specific T cells in both *in vivo* (Su, Peluso et al. 2002; Bozza, Perruccio et al. 2003) and *ex vivo* settings (Choudhury, Liang et al. 1999; Lodge, Jones et al. 2000; Lau, Wang et al. 2001). Unfortunately, the main drawbacks of using DCs include the amount of blood required to obtain sufficient numbers of DC precursors, the expense of the process due to the high concentration of cytokine cocktails required to grow them, the time consuming nature of the protocol and the necessary manipulation of DCs which is not ideal. Therefore in order to avoid *ex vivo* DC manipulation, strategies to manipulate them *in vivo* were initiated. Vectors carrying peptide or costimulatory molecule DNA sequences for *in vivo* DC transfection have been described (Mackensen, Herbst et al. 2000; Cerundolo, Hermans et al. 2004). Even though this branch of gene therapy is exciting and promising, it possesses all the drawbacks and fears of *in vivo* human gene transfection such as bio safety (Hacein-Bey-Abina, von Kalle et al. 2003), tumourogenicity and engraftment potential. Therefore there has been a large drive to develop and improve artificial systems to accomplish antigen presentation for immune gene therapy and immunomodulation in alternative safer ways. There are a number of cellular and non-cellular systems available to mimic artificial antigen presentation (Kim, Latouche et al. 2004). However despite successful strategies to by-pass defective antigen presentation and stimulate and

expand effector T cells *ex vivo* for therapeutic infusion as Adoptive Immunotherapy (Peggs and Mackinnon 2002; Peggs, Verfuert et al. 2003; Peggs and Mackinnon 2004), there is still no comprehensive artificial Antigen Presenting Cell (aAPC) system for both *ex vivo* and most importantly *in vivo* antigen specific T cell manipulation as Active Immunotherapy.

In order to address this, using nanotechnology, which uses nanoscale injectable, targeted and traceable "smart" devices capable of clinical important functions, we have developed and constructed an artificial nano-sized and super para magnetic targeted and traceable APC system capable of priming and expanding antigen specific T cells *in vivo*. These nano aAPCs were constructed using the latest generation of immune liposomes, approved for *in vivo* human use (Iden and Allen 2001; Maruyama 2002), by coating them with MHC Class I / peptide complexes and a range of adhesion, early activation, late activation and survival receptors as monoclonal antibodies and/or Fab regions at different ratios and combinations. These nano aAPCs possess the essential requirements for use *in vivo*: They are non-toxic, biodegradable, avoid fast recognition by the reticulo-endothelial system. They form immunological synapses with T cells (Prakken, Wauben et al. 2000; Giannoni, Barnett et al. 2005), which are essential for interaction. These liposomes are safe in terms of size (100nm; 50 times smaller than average cells), have good stability and favourable pharmacokinetic behaviour for safe *in vivo* trafficking. The artificial APCs are traceable *in vivo* via fluorescent and Magnetic Resonance Imaging (MRI). Super para magnetic liposomes also allow focussing of aAPCs to specific organs, tumour sites or body areas by applying external magnetic attraction. *Ex vivo* and *in vivo* models have been tested with encouraging results.

An important feature of the system is that when an artificial APC is tested in an *in vivo* setting, the aAPCs will also face the immune evasion and suppression mechanisms probably responsible for the disease itself or the malfunction of the patient's original APCs in the first place. Our system cannot be affected by most of these since it is not a cellular model. Therefore it cannot be influenced by immunosuppressive cytokines and cannot be infected by viruses as normal APCs are. Therefore no MHC class I and II down regulation is developed and they enter

the body as a “fully mature APC” that does not require further peptide processing, providing in this manner the number and amounts of signals required for successful APC - T cell interaction. Once all of the individual components have been produced in large batches, production of these sterile nano aAPCs in a ready to use form is achievable in less than 48 hrs and remain as such for at least 7 days. The system is capable of activating and expanding antigen specific T cells in a faster and superior manner than standard methods. Expanded T cells are fully functional; degranulate and produce cytokines when specifically challenged retaining all killing and secretory functional capabilities.

This aAPC system holds the potential promising use for *in vivo* clinical applications where there is a requirement for T cells to be specifically activated and expanded in the human body as a powerful novel active immunotherapy and cancer nanotechnology platform.

6 AIMS AND OBJECTIVES

The aims of this PhD will be to develop a novel targeted and traceable *ex vivo* and *in vivo* artificial antigen presenting cell system according to nanotechnology in order to use it for T cell adoptive and active immunotherapy:

- ❖ Activate:
 - ❖ Prime and expand antigen specific T cells in naïve individuals
 - ❖ Expand antigen specific T cells in memory individuals
 - ❖ Produce fully functional cells with relevant phenotypes, i.e. effector and memory
 - ❖ Examine different ratios, conditions and combinations to optimise activation and survival time of expanding T cells.
- ❖ Create collaborations with groups that can perform animal research in order to:
 - ❖ Look at the flow dynamics and presence of the system in an *in vivo* animal model
 - ❖ Test the feasibility and traceability of the system in an *in vivo* animal model

CHAPTER 2

MATERIALS AND METHODS

2.1 INTRODUCTION

One of the main goals of this PhD project was the development of an artificial APC aimed for both *ex vivo* and *in vivo* human clinical applications against tumours and viral diseases. Therefore the system needed to fulfil the nanotechnology requirements (NIH 2005) previously described in the introduction. The strategies and the logistic of the creation of the system were very challenging since the development and construction of artificial APCs using liposomes had never been described before. For this reason the construction of the super-para-magnetic nano artificial APC is described as a separated chapter (Chapter 3). The rest of the materials and methods are described below. In general terms, in order to create this artificial APC system, three main components were required:

- ❖ 1) Immunoliposomes, which are targeted vectors carrying the immunological signals.
- ❖ 2) MHC class I / peptide complexes (referred to herein as monomers), which deliver the antigen specific signal (also called “signal one”) and
- ❖ 3) Fab antibody regions or specific monoclonal antibodies (mAb) for the different adhesion, co-activatory and survival receptors, which deliver essential secondary signals.

Once these three essential components were generated, the aAPCs were created by coating the nano liposomes with monomers, mAbs and/or Fab regions (Chapter 3). The functionality of the aAPCs was then assessed using different stimulation conditions and functional assays.

2.2 IMMUNOLIPOSOMES

2.2.1 Preparation Of Immunoliposomes

Liposome construction took place as follows: synthetic lipids were reconstituted from powder into a final lipid mix (see below) using chloroform (AnalarR BDH) as organic solvent (fig 2.1A). Once the mix was created, chloroform was eliminated by evaporation (fig 2.1B), which was accomplished in minutes using a Büchi rotary evaporator R-200 leaving a dry lipid film (figure 2.1C). The dry lipid film was hydrated in order to form multilayer “onion” vesicles, which are formed passively (figure 2.1C). The hydration buffer [Hepes (Sigma-Aldrich) 25 mM + NaCl (AnalarR BDH) 140 mM; pH 7.4 filtered and degassed] creates their liquid interior. Liposomes can be generated to different sizes. Using two 1ml syringes, which are part of a mini extruder kit (Avanti Polar Lipids INC, figure 2.2), the multilayer “onion” vesicles were passed back and forward 20 times through a 100 nm membrane. At the end of the process, millions of nano liposomes of 100 nm were created. To check the correct size and spherical shape, liposomes were analysed using scanning electron microscopy (figure 2.3), which confirmed the size, shape and approximate number of liposomes. Liposomes were then stored at 4°C and retained their size and quality for two weeks. Following this time, they start to lose the liquid interior and begin to leak due to hydrolysis of the lipids (Gregoriadis 1993).

Five different lipids (purchased from Avanti Polar Lipids, INC) were used for the lipid final mix: Phosphatidylcholine (PC); Cholesterol (Chol); 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N- [Methoxy (Polyethylene glycol)-2000] (DSPE-PEG); 1,2-Distearoyl-sn-Glycero-3 Phosphoethanolamine N [Maleimide (Polyethylene glycol) – 2000] (DSPE-PEG-MAL) and [1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine-N- (Lissamine Rhodamine B Sulfonyl)] DPE-RHODAMINE. They were mixed in the following molar ratio:

PC:CHOL:DSPE-PEG:DSPE-PEG-MAL 2:1:0.02:0.08

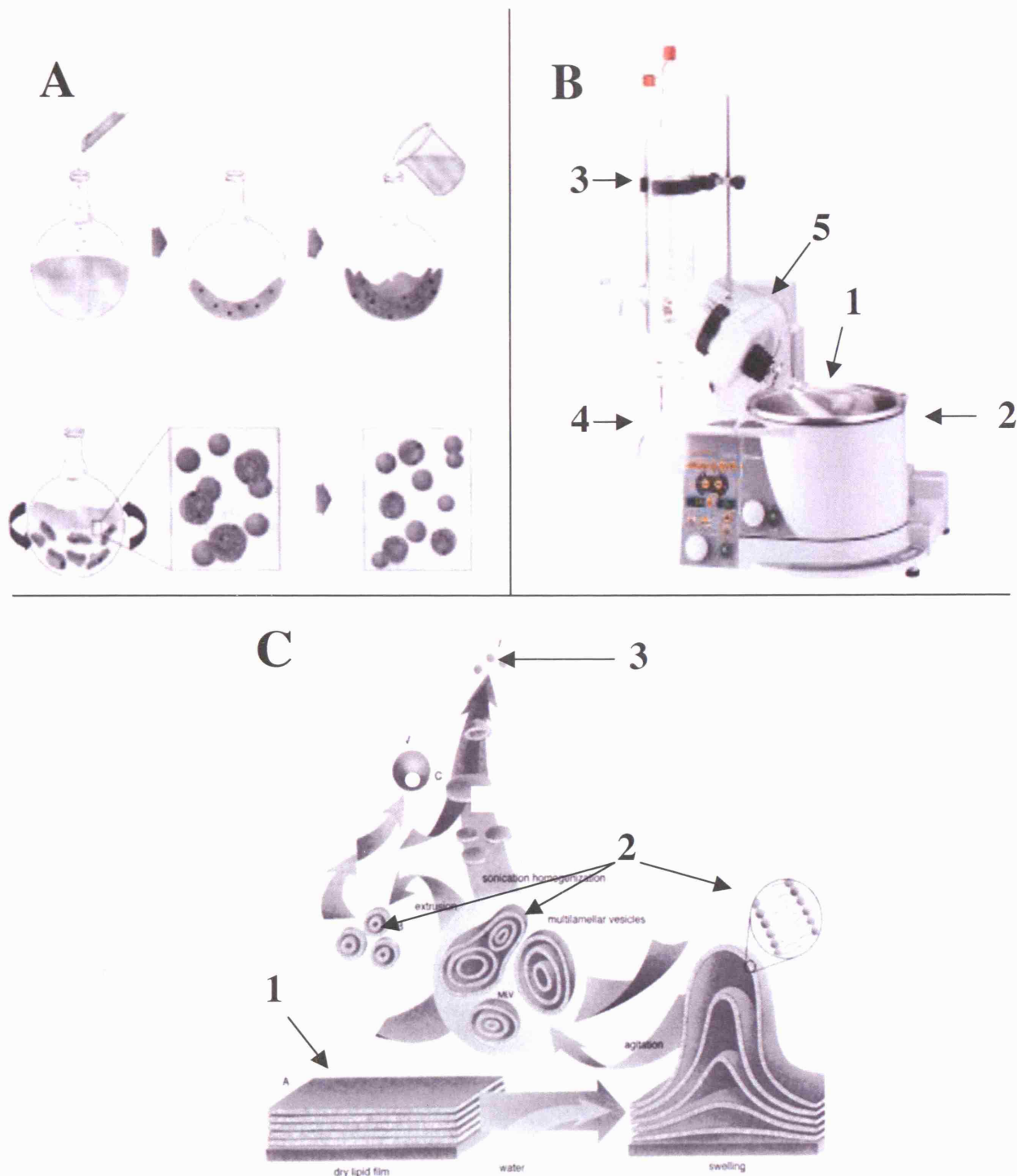


Figure 2.1.

PREPARATION OF IMMUNOLIPOSOMES: A.- Dissolution of lipids in chloroform. B.- Rotary evaporator: 1 flask containing dissolved lipids in chloroform. 2 water bath. 3 liquid nitrogen container. 4 waste. 5 rotation device. C.- After chloroform evaporation, lipid films are left (1). They were then hydrated and multilayer or multilamellar (MLV) “onion” vesicles (2) containing the bilayer membranes were then formed by vortex agitation or sonication. These multilayer vesicles were then passed through a mini extruder to give the desired liposome final size (3). Figures: Courtesy of Avanti Polar INC.

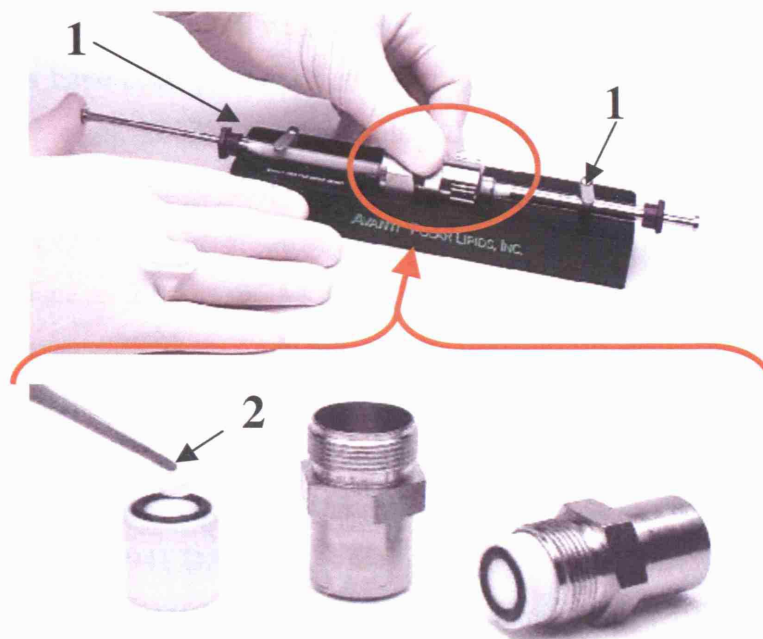


Figure 2.2.

MINI EXTRUDER: The multilayer “onion” vesicles contained in 1 ml syringes (1), were passed 20 times back and forward through a 100 nm membrane (2) to produce the 100 nm nano-liposomes. Figure: Courtesy of Avanti Polar INC.

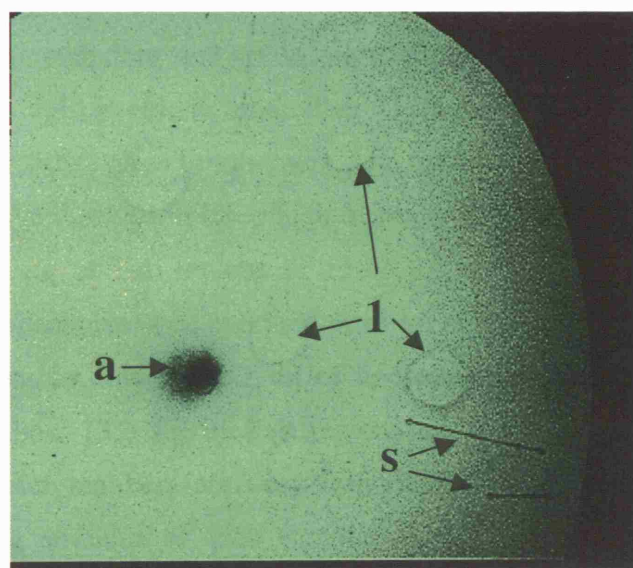


Figure 2.3.

SCANNING ELECTRON MICROSCOPE IMAGE OF LIPOSOMES: Size (100 nm), structure (spherical), homogeneity and approximate number of liposomes (1) was confirmed by electron microscopy after preparation. **a.-** artefact (screen burn). **s.-** size markers.

The Rhodamine fluorescent lipid was added at 1% of the PC amount. These amounts and ratios have been previously standardised (Pagnan, Stuart et al. 2000; Pastorino, Brignole et al. 2003). The function and relevance of each lipid in the constructs will be explained in chapter 3. However the details concerning their construction are explained here. According to their molecular weights, the preparation took place as follows in 25 ml of chloroform:

PC: MW 760 DA76 mg
DPE-RHODAMINE.....0.76 mg
Chol: MW 386.7 DA.....19.33 mg
DSPE-PEG: MW 2805 DA.....4.20 mg
DSPE-PEG-MAL: MW 2941 DA....11.76 mg

Following these published standards, antibodies were mixed in a 1 : 2000 (Ab : Mal lipid molar ratio) and Fab regions in a 1 : 1000 (Fab : Mal lipid molar ratio). MHC class I monomers had not been described previously bound to maleimide liposomes. However since MHC class I monomers and Fab regions have very similar molecular weights (40KDA and 50KDA respectively), a 1 : 1000 (MHC class I monomer : Mal lipid) molar ratio was therefore also chosen for them. Following these standard recommendations and according to the previously mentioned molar ratios, 1 mg of MAL lipid is able to bind either 25.5 µg of antibody, 17 µg of Fab regions or 13.6 µg of MHC class I monomers. The final hydrated mix of liposomes contains 11.76 mg of maleimide lipids, which therefore can bind a total of 299.6 µg of antibodies, 199.6 µg of Fab regions or 159.6 µg of MHC class I monomers respectively. These liposomes were hydrated in 15 ml of Hepes Buffer final volume and split in 1 ml batches. Therefore 1 ml of liposomes can bind a maximum of 19.97 µg of antibodies, 13.3 µg of Fab regions or 10.64 µg of MHC class I monomers. These later numbers are very important since as discussed later in chapter 4, increasing amounts of each signal (without exceeding the maximum liposome binding capacity) were chosen to titrate and coat liposomes in order to check the functionality of the system. For instance, the maximum liposome binding capacity of MHC class I monomers is 10.64 µg per ml of liposomes. Therefore liposomes were coated with increasing amounts of monomers: from 2.66 µg to 3.77

μg , 4.54 μg , 5.32 μg , 7.98 μg and 10.64 μg , which were 25%, 35%, 42%, 50%, 75% and 100% respectively of the liposome maximum monomer binding capacity. In order to have an estimate of the amount of molecules bound per liposome, the molecular weights of MHC class I molecules, mAbs and Fab regions were converted into grams. Any molecular weight in Daltons can be converted into grams by multiplying Daltons by the constant 1.650×10^{-24} . By multiplying the molecular weights of the interest proteins, which are 40,000 DA for an MHC class I peptide complex without the transmembrane region of the molecule; 150,000 DA for a monoclonal antibody and 50,000 DA for a Fab region by this constant, the amount of molecules were estimated. According to this, 1 μg of MHC peptide complexes equals 1.5×10^{13} molecules. 1 μg of mAb equals 4.04×10^{12} molecules and 1 μg of Fab regions equals 1.21×10^{13} molecules. Because of the small size of the liposomes and the large number generated, the estimation of the exact numbers of liposomes measured by electron microscopy and FACS analysis are only an approximate. We calculated an approximate amount of 250 million liposomes per ml. Therefore we can extrapolate that a single liposome is covered with an approximate number in the range of 150,000 to 600,000 MHC class I molecules depending on the amount added to the liposomes (from 2.66 μg to 10.64 μg as discussed previously). The surface area of a 100 nm liposome (sphere) is $31.4 \mu\text{m}^2$ and was calculated as follows: $4\pi(r)^2 \Rightarrow 4 \times 3.14 \times (50)^2 = 31400 \text{ nm}^2 = 31.4 \mu\text{m}^2$. According to this, liposomes possess thousands (from 5,000 to 19,000) of MHC molecules per μm^2 of surface. The normal size of the immunological synapse between T cells and APCs is quite variable. However studies show that the size of the synapse is about $12.5 \mu\text{m}^2$ (Grakoui, Bromley et al. 1999; Gascoigne and Zall 2004). Therefore 100 nm liposomes have enough surface area ($32.4 \mu\text{m}^2$) to form and maintain immunological synapses ($12.5 \mu\text{m}^2$). Some studies had suggested that a density of 60 MHC class I molecules/ μm^2 or more was enough to trigger T cell activation, proliferation and cytokine production (Qi, Groves et al. 2001), however it has been demonstrated that a single MHC peptide complex is sufficient to trigger the T cell receptor (Irvine and Davis 2002). Therefore in order to investigate the functional properties of our system, it was needed to be functionally tested.

Theoretically however, we had generated a system with all the essential requirements including size and density of molecules to work as an antigen presenting cell.

2.2.2 Chromatographic Purification

In order to purify and separate unbound MHC/peptide complexes, mAbs and Fab regions from the effectively coated liposomes, the samples were chromatographed using sepharose CL-4B beads (40-165 μm bead diameter) from Sigma-Aldrich packed in an XK 16mm/20cm (Diameter/length) column from Amersham Biosciences. Following protein-liposome coupling (chapter 3), the liposome – protein samples were taken to a 2 ml volume using Hepes hydration buffer (section 2.2.1) and chromatographed in 30 minutes in order to recover coated liposomes exclusively.

2.2.3 Concentration, Filtration And Storage

Following coated liposome chromatographic purification. The coated liposome fractions (2 fractions of 3 ml each) were recovered and concentrated down to a final 3 ml volume using Centricon Plus-20 / 100,000 MWCO tubes from Amicon-Millipore and then filtrated using single use non-pyrogenic 0.45 μm filters from Minisart Santorius. Coated liposomes were then ready to use or were store at 4°C to up to 7 days.

2.2.4 Dot Blot

Dot Blots were performed in order to reveal the presence or absence of peptides or proteins in biological samples. For instance, a dot blot can reveal whether MHC peptide complexes are well refolded, whether they are biotinylated and whether they are present on the surface of liposomes as explained in detail in chapter 3. The protocol however is explained here. A nitro-cellulose membrane (Hybond ECL-

Amersham Biosciences) pre-wetted in transfer buffer (10 ml of Methanol from AnalarR BDH, 250 µl of SDS 10%, 5 ml of transfer buffer and 35 ml of H₂O) was placed onto a vacuum manifold system. Samples were loaded or “dotted” on the membrane and then blocked using 1% BSA from Sigma-Aldrich in PBS (AccuGENE-Cambrex) for 1hr. Detection antibodies such as W6/32 and MA2.1 were added in 1:1000 dilution for 1 hr after washing of the membrane with 0.5% Tween (AnalarR BDH) PBS Buffer. A second antibody (anti mouse peroxidase) was added to allow detection, which is carried out using Amersham Biosciences ECL detection kit. Equal volumes of the two kit solutions were added to the membrane for 1 min to excite the peroxidase detection antibodies attached to the samples, which were detected as dark dots by Kodak 100 NIF 18 x 24 cm films. 5, 10, 15, 30, 45, 60, 120 and 300 second exposure times were performed to reveal the results (see figures 3.9 and 3.10 in chapter 3).

2.3 MHC/PEPTIDE COMPLEX SYNTHESIS

2.3.1 Preparation Of MHC Class I Monomers

As a proof of principle and because our group has previous experience with the cytomegalovirus (CMV) natural immune response, we chose the A2/NLV (pp65-CMV immunodominant peptide purchased from Alta Bioscience) system as first model. The full sequence of this peptide is NLVPMVATV, letters that stand for the following amino acids in respective order: asparagine, leucine, valine, proline, methionine, valine, alanine, threonine and valine. cDNA encoding the extra cellular sequence of the HLA-A*0201 molecule was cloned into the pET-3d vector (Invitrogen), which was later inserted into BL21pLys bacteria cells for protein expression, whereas the β_2m cDNA cloned into the PHN1 vector (kindly obtained from Dr Wiley at Harvard University) was inserted into *Escherichia coli* XA90 strain for protein expression. Cloning and protein expression were induced by growing the bacteria in 1 litre of XYT medium (10 g tryptone from Sigma-Aldrich, 5 g yeast extract from Sigma-Aldrich, 5 g NaCl from AnalarR BDH) containing 1 g

of glucose (AnalarR BDH) plus 50 mg of ampicilin (Sigma-Aldrich) and 34 mg of chloramphenicol from Sigma-Aldrich (the latter was only added to the A2 protein culture). Antibiotics prevent the growing of bacteria not carrying the plasmid since the plasmid encodes the A2 and β_2m protein sequences together with antibiotic resistance. Bacteria cultures were incubated in constant mixing at 37°C until they reached the mid-logarithmic exponential expansion phase, identified when the optical density (OD) at 550 nm reached approximately 0.6 OD. Then 1 mg of IPTG (Alexis Biochemicals) was added for additional 4 hr in order to induce protein expression. Cells were then harvested by centrifugation (4000 g for 25 min at 4°C) and in order to recover the protein of interest, bacteria were lysed with 10 ml of a hyperosmolar lysis buffer (Tris-HCL 50 mM from AnalarR BDH, 25% sucrose from AnalarR BDH, 1 mM EDTA from Gibco-Invitrogen, pH 8) and recovered again by 10 more minutes centrifugation at 4000 g. 10 ml of Per-B bacterial protein extraction reagent (Pierce Perbio) plus 25 mg of lysozyme (Sigma-Aldrich) were added to continue the lysis, further accomplished by 10 cycles (15 seconds each) of sonication. To recover the A2 and β_2m proteins, samples were washed once with 10 ml of detergent buffer (NaCl 0.2 M, deoxycholic acid 1%, nonidet P40 1%, Tris-HCL 20 mM, EDTA 2 mM, pH 7.5) plus three further washes with 10 ml of Triton buffer (Triton X100 0.5% from AnalarR BDH, Tris-HCL 50 mM, NaCl 100 mM, EDTA 1 mM, pH8) and centrifuged at 10,000 rpm for 20 min between each wash. The resulting A2 and β_2m pellets were resuspended in 5 ml of final resuspension buffer (Tris-HCL 50 mM, EDTA 1 mM, DTT 1 mM). Proteins then were solubilised overnight in urea buffer (8 M Urea from AnalarR BDH, 0.1 M NaH₂PO₄, 0.01 M Tris pH 8, 0.1 mM EDTA, 0.1 mM DTT), the quality was assessed by SDS-PAGE gel (figure 2.4) and the quantity determined by BCA (Pierce Perbio) protein assay (figure 2.5). Proteins were then aliquoted, stored at –70°C for future use or used immediately to generate MHC/peptide complexes (section 2.3.4). The C-terminal end of the A2 heavy chain in these constructs has been modified to include a biotinylation site, which can be later recognised by the BirA enzyme to produce biotinylated monomers, which are then used to create tetramers (section 2.3.5). However, monomers do not need to be biotinylated to be

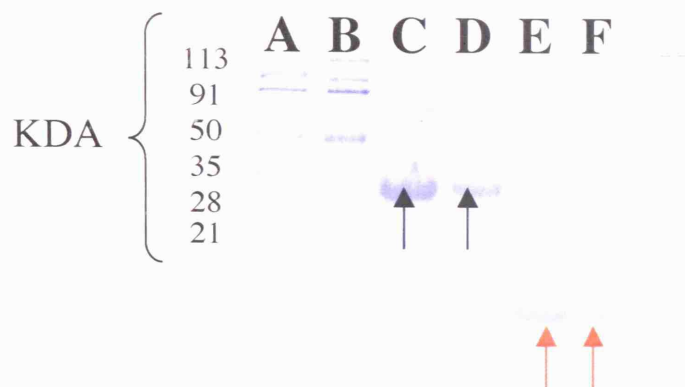


Figure 2.4.

SEPARATION OF INCLUSION BODIES BY SDS-PAGE: To assess the correct protein expression after bacteria cloning and harvesting, proteins were run on SDS gel to confirm their presence and size. **A** and **B** are the molecular weight standards. **C** and **D** show the A2 heavy chain in duplicate (black arrows at ~30KDA). **E** and **F** show the β_2 m protein in duplicate (red arrows at ~11KDA).

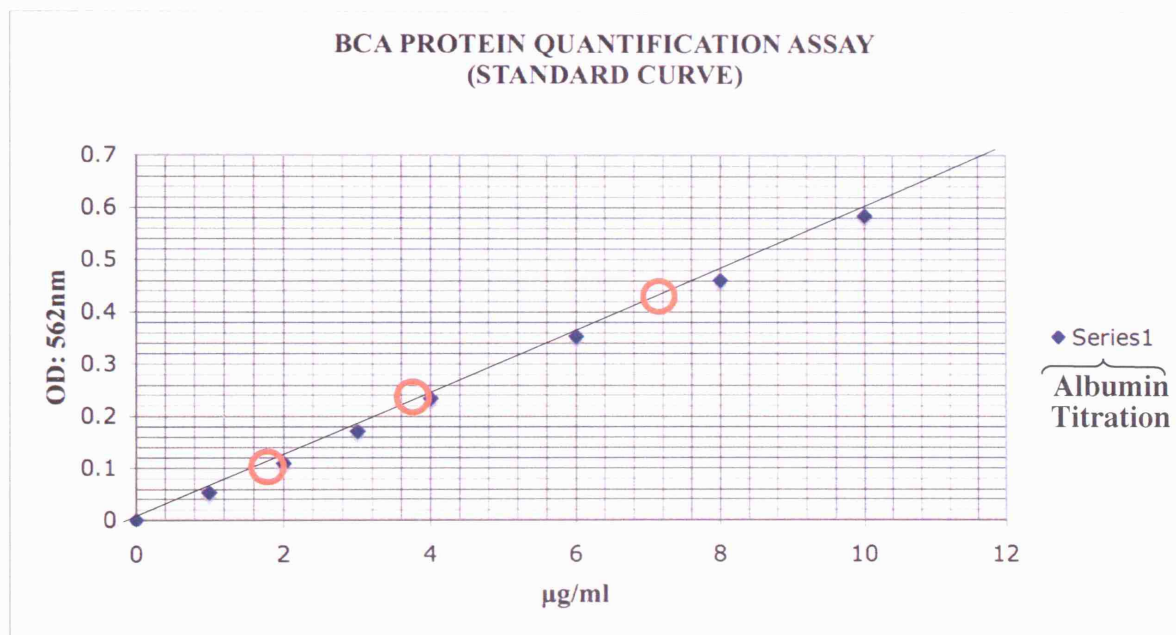


Figure 2.5.

BCA PROTEIN ASSAY: In order to calculate the concentration of any given protein. A standard curve of a known protein concentration (albumin) was first plotted (blue dots). The test protein was then plotted over the curve according to the OD read-out given by the spectrophotometer at 562 nm. For example: 1, 2 and 4 μ l of a test protein were analysed. Their respective spectrophotometer read-outs were 0.1, 0.2 and 0.4 nm (red circles). Once plotted, the 1 μ l sample = 1.8 μ g/ml, the 2 μ l sample = 3.6 μ g/ml and the 4 μ l sample = 7.2 μ g/ml. All measurements multiply by a constant: 500 for the 1 μ l sample, 250 for the 2 μ l sample and 125 for the 4 μ l sample. Therefore 1.8 μ g/ml x 500 = 900 μ g/ml, 3.6 μ g/ml x 250 = 900 μ g/ml and 7.2 μ g/ml x 125 = 900 μ g/ μ l giving an average of 900 μ g/ μ l, which gives a final 0.9 μ g/ μ l total protein concentration.

attached onto liposomes, which (as explained in chapter 3) is a great advantage of the system in terms of cost-effectiveness and also in low immunogenicity.

2.3.2 SDS-PAGE Gel

The correct size of the A2 (~30 KDA) and β_2m (~11 KDA) was assessed by running 5 μ g of the recovered protein + equal volume of SDS loading buffer (50 mM Tris pH 6.8, SDS 2%, 0.1% bromophenol blue from Sigma-Aldrich, 10% glycerol, 100 mM dithiotreitol) in parallel with two molecular weight standards (Bio-Rad Laboratories) for 1hr at 150 volts and 400 mA. Samples were heated for 3 minutes at 90°C before loading to denature the proteins. The gel was then stained with coomassie blue for at least 20 minutes, de-stained and dried (Gel dryer model 583 from Bio-Rad Laboratories) for analysis (figure 2.4).

SDS-PAGE Gel Formula:

- ❖ Lower Gel: 0.375 M Tris pH 6.8, acrylamide 30% (Bio-Rad laboratories), 0.1% SDS, 0.03% ammonium persulfate, 0.15% TEMED (BDH)
- ❖ Upper Gel: 0.125 M Tris pH 6.8, acrylamide 5%, 0.1% SDS, 0.1% ammonium persulfate, 0.1% TEMED
- ❖ Running Buffer: 25 mM Tris, 250 mM glycine, 0.1% SDS.

2.3.3 BCA Protein Quantification Assay

Bicinchoninic acid (BCA) allows the colorimetric detection and quantification of total protein by spectrophotometer absorbance at 562 nm. Protein concentrations were determined and reported with reference to standards of a protein at known concentration such as bovine serum albumin (BSA). A series of dilutions of albumin were prepared and assayed and compared alongside the test protein based on a standard curve. To prepare the albumin standard, a titration curve was created by aliquoting from 0.5 μ g to 5 μ g of albumin in 500 μ l of H₂O. The test protein was also aliquoted in 500 μ l of H₂O using 1, 2 and 4 μ l of the bulk sample. 500 μ l from the BCA A, B and C solutions mix in a 1:0.96:0.04 volume ratios were added to all

the samples. Samples were vortexed and incubated at 60°C for 1 hr to allow colorimetric development. Samples were analysed by spectrophotometer and final protein concentration measured according to the standard curve (figure 2.5).

2.3.4 MHC/Peptide Complex Refolding

As mentioned previously, following expression, purification, recovery, solubilization and quantification of the A2 and β_2m proteins, the peptide of interest was added to make MHC peptide complexes. The standard refolding buffer conditions are as follows:

9.3 mg of A2; 4.8 mg of β_2m and 2 mg of the relevant peptide (in this case the pp65-NLVPMVATV * CMV peptide (Alta Biotech), were left to refold at 4°C in 200 ml refolding buffer (100 mM Tris-HCL pH 8; 400 mM L-arginine HCL from Sigma-Aldrich; 2 mM EDTA; reduced glutathione 0.307 gr from Sigma-Aldrich; oxidised glutathione 0.061 gr from Sigma-Aldrich and 180 ml of d_4H_2O ; pH 8) for 48 hrs with constant stirring. β_2m and the peptide were added first followed by the A2 molecule to prevent aggregation of the A2 MHC heavy chains and improve the MHC/peptide complex refolding.

After 48 hrs, the 200 ml refolding sample was spun down 20 min at 3300 rcf/4°C to remove aggregated protein and the supernatant was then concentrated down to 2 ml using a combination of stirred ultra filtration cell amicon (Millipore) system and Vivaspin 10,000 KDa cut-off tubes (Vivascience). Low salt buffer exchange (10 mM Tris-HCL, 5 mM NaCl) was performed to prepare the sample for future biotinylation and Fast Protein Liquid Chromatography (FPLC). FPLC recovers and purifies the MHC peptide complexes generated and provides a profile of the refolding (figure 2.6), which includes the efficiency of the refolding, quality of the monomers, approximate amount and purity of the constructs. The fractions where the correct refolded MHC/peptides complexes were located were recovered and concentrated down to 1 ml and stored at 4°C for either short-term use, biotinylation (section 2.3.5) or stored at -20°C for long-term future use.

2.3.5 Biotinylation And Tetramer Synthesis

Following the generation and concentration of the MHC peptide monomers as described previously, samples were biotinylated by incubating the desired amount of monomers with Bir A enzyme (Avidity LLC) for 2 hrs in a water bath at 30°C plus 25 µl of biomix A (0.5 M bicine buffer pH 8.3); 25 µl of biomix B (100 mM ATP, 100 mM magnesium acetate, 400 µM d-biotin from Sigma-Aldrich); 10 µl of protease inhibitors mix (leupeptin 5 mg/ml and pepstatin 2.5 mg/ml) and 5 µl of biotin. The sample was then passed again through the FPLC column, this time in physiological salt buffer (10 mM Tris-HCL, 150 mM NaCl) in order to purify the biotinylated monomers (figure 2.7). The amount of monomers was assessed by BCA assay and biotinylation efficiency was assessed by native gel as shown in figure 2.8 and explained in section 2.3.6. Biotinylated monomers were then ready to be tetramerized. Tetramers were generated by mixing biotinylated monomers at 4°C degrees in the presence of fluorescent phycoerythrin (PE) conjugated streptavidin (Sigma-Aldrich) at a 1:4 streptavidin : monomer molar ratio. Streptavidin possesses four biotin-binding sites and it is added in 1/10th of the corresponding total amount every 20 minutes to improve the proportion of tetramers formed. Tetramers were then stored at -20 or -70°C until further use. Tetramers were used as a specific detection tool for antigen specific T cell populations using Flow cytometry as described later in chapter 4.

2.3.6 Native Gel

The efficiency of biotinylation was assessed by running 3 µg of the purified biotinylated monomers on a native gel (150 V / 400 mA for 1 hr) in different conditions: non-biotinylated monomers themselves, non-biotinylated monomers incubated with 3 µl of streptavidin for 1 hr, biotinylated monomers themselves and biotinylated monomers incubated with 3 µl or 6 µl of streptavidin for 1 hr. All the samples had an equal volume of native gel loading buffer (50 mM Tris pH 8.8, 0.1% bromophenol blue from Sigma-Aldrich, 10% glycerol). The gel was then

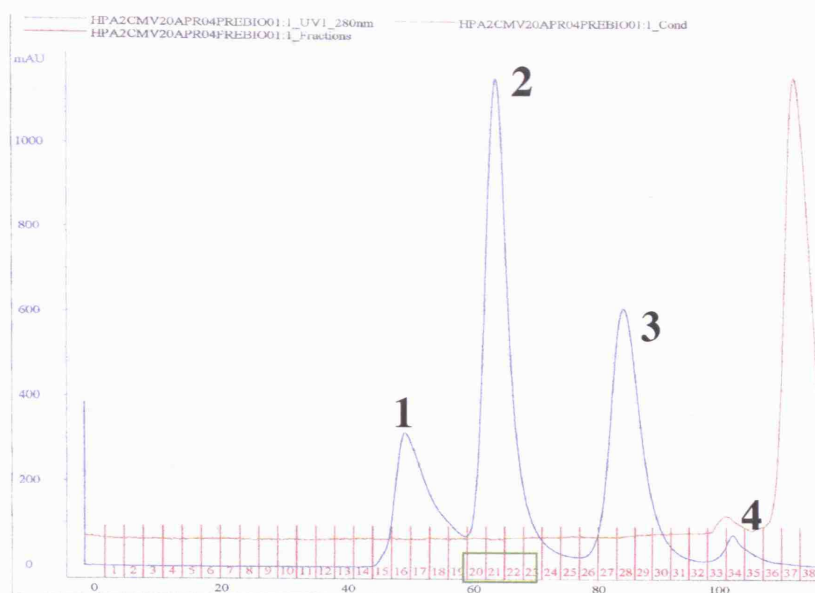


Figure 2.6.

MHC CLASS I MONOMER FPLC PROFILE: Following 48 hrs of A2, β_2m and peptide refolding induction, the sample was concentrated down and passed through an FPLC column to assess the approximate amount, purity and quality of the newly formed MHC class I peptide complexes. 1.- protein aggregate. 2.- correct refolded MHC/peptide complexes. 3.- free β_2m . 4.- free peptide. Fractions 20 to 23 were recovered.

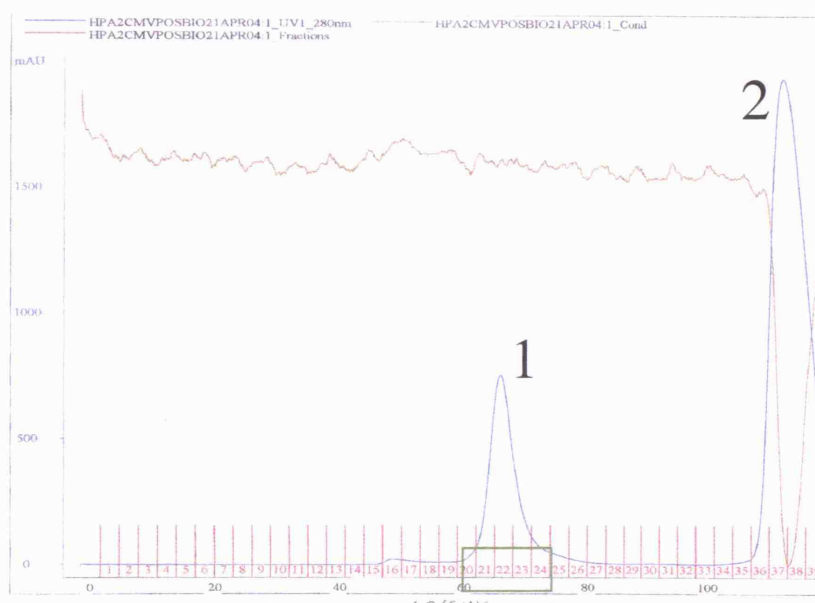


Figure 2.7.

BIOTINYLATED MHC CLASS I MONOMER FPLC PROFILE: Following biotinylation, monomers were passed through an FPLC column to remove extra free biotin (2) and purify the biotinylated monomers (1). Fractions 20 to 24 were then recovered.

stained with coomassie blue for at least 20 minutes, de-stained and dried for analysis. By detecting a shift in the biotinylated monomers + streptavidin samples when compared with controls, the efficiency of biotinylation was assessed (fig 2.8).

Native Gel Formula:

- ❖ Lower Gel: 0.375 M Tris pH 8.8, acrylamide 8% (Bio-Rad Laboratories), ammonium persulphate 0.03%, 0.003% TEMED (BDH)
- ❖ Upper Gel: 0.125 M Tris pH 6.8, acrylamide 5%, ammonium persulphate 0.03, 0.003% TEMED
- ❖ Running Buffer: 24.8 mM Tris, 192 mM glycine. Once the gel is polymerised, it is pre-run for 30 min at 150 volts to remove any salt excess.

2.4 GENERATION OF FAB REGIONS

The use of Fab regions rather than whole antibodies is preferable since the Antibody-Fc regions are highly immunogenic (Allen 2002). Monoclonal antibodies raised against adhesion, costimulatory and survival receptors were purchased from BD Pharmigen or Diaclone Research unless otherwise stated. The anti-human CD28 was obtained as a gift from Dr Mark Frewin at Oxford University. Some of these mAb had to be further purified before being digested by papain enzyme (Pierce Perbio) in order to obtain the Fab regions. None of these antibodies are super agonists. This means that are not capable of inducing T cell activation in the absence of TCR ligation (Luhder, Huang et al. 2003).

2.4.1 Antibody Purification

Purification of antibodies was accomplished by passing them through a protein “A” column (Bio-Rad Laboratories), which binds the Fc regions of the antibodies, therefore allowing considerable amounts of washing buffer to pass through and remove non-relevant proteins and debris (figure 2.9). Following Ab elution from the column, BCA protein quantification was performed as described in section 2.3.3 and antibodies were either stored at -20°C or digested as described below.

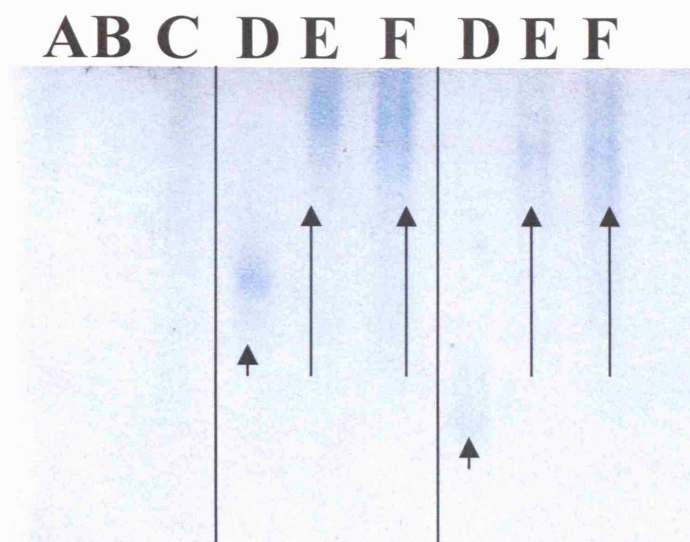


Figure 2.8.

ASSESSMENT OF BIOTINYLATION EFFICIENCY BY NATIVE GEL: Streptavidin possess biotin binding sites and runs slower in the gel when attached to biotin and even slower when attached to biotinylated proteins such as monomers as shown above. The shift shown by the big arrows in lines **E** and **F** confirmed a 100% biotinylation of the monomers. **A.-** Streptavidin running by itself. **B.-** Non biotinylated monomers by themselves. **C.-** Non biotinylated monomers incubated with 3 μ l of streptavidin for 1 hr. **D.-** Biotinylated monomers by themselves. **E.-** Biotinylated monomers incubated with 3 μ l of streptavidin and **F.-** Biotinylated monomers incubated with 6 μ l of streptavidin also for 1 hr.

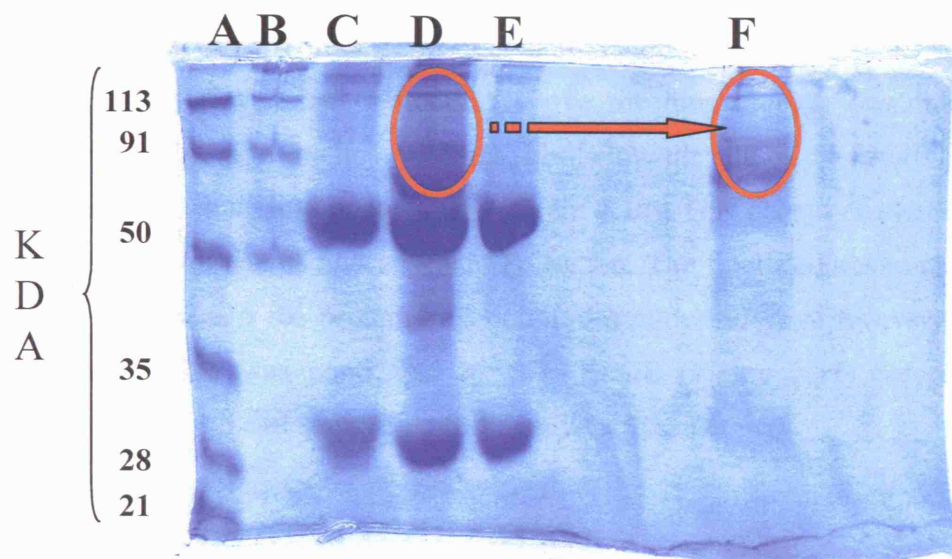


Figure 2.9.

ANTIBODY PURIFICATION GEL TO CHECK PROTEIN "A" COLUMN PURIFICATION: Some Abs need to be purified before their *in-vitro* and *in-vivo* use by passing them through a protein "A" column, which binds the Abs via their FC regions. **A** and **B**.- Molecular weight standards **C**.- Control (an already pure commercial antibody). **D**.- Antibody of interest un-purified. **E**.- Antibody of interest following protein "A" column purification. **F** and **red ovals**.- Non-relevant proteins and debris following antibody purification.

2.4.2 Antibody Digestion

Antibodies were digested with papain for 5 hrs following the Pierce Perbio standard protocol (PIERCE). Papain is considered the enzyme of choice for the preparation of Fab regions (Rousseaux, Rousseaux-Prevost et al. 1983) whereas pepsin is the enzyme of choice for the generation of Fab₂ fragments (Figure 2.10). Papain was fixed onto a membrane in order to avoid contamination of the final product with the enzyme. Papain cleaves IgG antibodies above the hinge region containing the disulfide bonds that join the heavy chains, but below the site of the disulfide bond between the light chain and heavy chain (figure 2.10). This generates two separate monovalent Fab fragments and an intact Fc region. The final Fc-Fabs sample mix was again run through the protein “A” column to allow selective recovery of the Fab regions, which was confirmed by SDS-PAGE (Figure 2.11) before BCA protein quantification and storage at -20°C until further use.

2.5 STIMULATION CONDITIONS

2.5.1 Samples

Blood from HLA A*0201^{positive} / B*0702^{negative}, CMV positive and negative individuals was obtained from healthy volunteers. All individuals were informed of the study and consent was obtained. All volunteers were HLA typed at the allelic level by the histocompatibility laboratory at the Anthony Nolan Trust. We chose A2^{positive} individuals due to its prevalence in the Caucasoid population (50%). Volunteers had to be B7^{negative} since A2^{positive} B7^{positive} individuals present pp65 peptides predominantly by B7 MHC molecules rather than the A2 molecules (Lacey, Villacres et al. 2003), whereas A2^{positive} B7^{negative} individuals mount anti pp65 CMV responses predominantly by A2 MHC molecules.

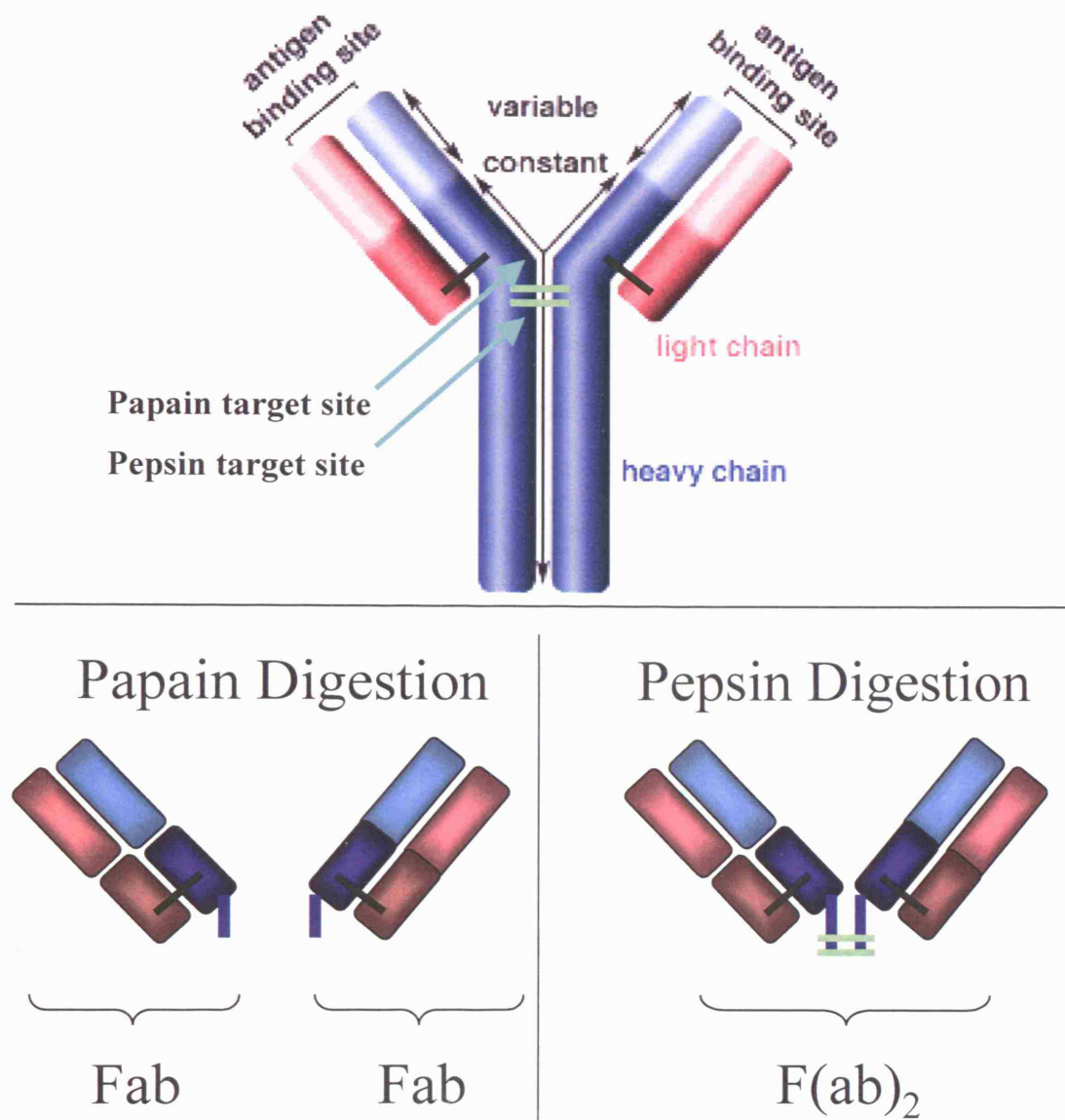


Figure 2.10.

ANTIBODY ENZYMATIC DIGESTION: Papain is the enzyme of choice for the generation of Fab regions whereas pepsin is the enzyme of choice for the generation of F(ab)₂ fragments. Papain cleaves IgG antibodies above the hinge region (green bonds) containing the disulfide bonds that join the heavy chains, but below the site of the disulfide bond between the light chain and heavy chain (black bonds) generating two separate Fab regions. Pepsin however, cleaves the antibody below the hinge region producing one F(ab)₂ fragment.

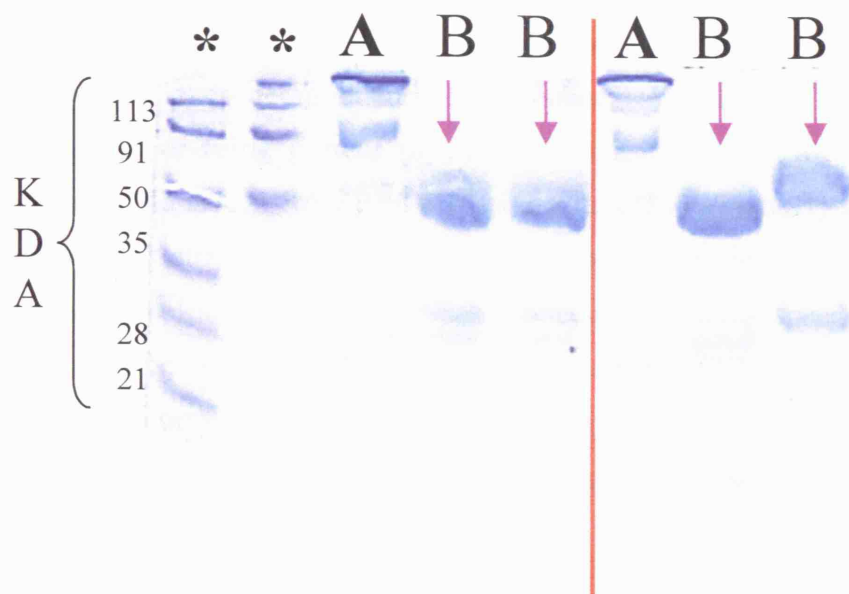


Figure 2.11.

SEPARATION OF FAB REGIONS BY SDS-PAGE:

Following papain Ab digestion, Fab regions were separated from the Fc regions using a protein "A" column and checked by SDS-PAGE before storage. *.- Molecular weight standards. **A.**- Whole Ab. **B.**- Fab Regions. Fab fragments run at 50 KDa on an SDS-PAGE.

2.5.2 Peripheral Blood Mononuclear Separation

Peripheral Blood Mononuclear Cells (PBMCs) were isolated by density centrifugation over lymphoprep (Nycomed) using standard protocols: an equal volume of blood was layered over an equal volume of lymphoprep (Cedarlane labs, Canada) in a sterile 20 ml universal tube (Bibby Sterilin). The tubes were then centrifuged at 2200 rpm for 20 minutes without the brakes applied to prevent the lymphocyte layer being disturbed. Lymphocytes were then collected from the interface layer using pasteur pipettes and transferred into a 50 ml Falcon tube (Becton Dickinson, USA). RPMI 1640 medium (BioWhittaker) was added and the cells were centrifuged at 1800 rpm for 10 minutes to remove any contaminating lympholyte solution. Cells were washed once more and resuspended in 10 ml RPMI 1640 medium containing 10% Foetal Calf Serum (FCS), 1 U/ml penicillin and 1 µg/ml streptomycin (BioWhittaker) in preparation for counting (section 2.5.2.1). PBMCs were preferentially used fresh but were also cryopreserved (section 2.5.2.2).

2.5.2.1 Cell Counting And Viability

Cells were counted using the Trypan Blue exclusion method. A 10 µl aliquot of cells was mixed with 10 µl of 0.4% Trypan Blue solution (Sigma-Aldrich) and transferred to a haemocytometer counting chamber (0.1 mm depth, Webster Scientific International, UK). The number of live and dead cells was determined by using a phase contrast microscope (DMLB, Leica) where the live cells appear translucent whereas the dead cells appear blue.

2.5.2.2 Cell Cryopreservation

Cells to be cryopreserved were centrifuged at 1600 rpm for 5 minutes. The supernatant was discarded and the cells resuspended in a freezing solution consisting of 90% FCS and 10% DMSO Dimethylsulphoxide (AnalarR BDH).

Cells were pipetted into 1.5 ml cryotubes (Nunc, Denmark) containing at least 5×10^6 cells, these aliquots were rapidly transferred to -80°C overnight and then transferred into liquid nitrogen for long-term storage.

2.5.2.3 Thawing Cryopreserved Cells

An aliquot was taken from the liquid nitrogen storage and rapidly thawed at 37°C to prevent the formation of ice crystals that could damage the cells. Cells were transferred into a 50 ml Falcon tube containing 50 ml of RPMI 1640 and centrifuged at 1600 rpm for 5 minutes to remove the freezing solution. Cell counts were performed and cell viability assessed.

2.5.3 CD8 Positive T Cell Sorting

CD8 positive T cells were sorted by positive selection using Miltenyi Biotech Micro Beads. Following PBMCs isolation as described in section 2.5.2, the cell pellet was resuspended in 80 μl of PBS buffer per 10^7 or less total cells and incubated for 15 minutes at $4-8^{\circ}\text{C}$ with 20 μl of MACS CD8 Micro Beads. Following this period, cells were washed and resuspended in 500 μl of PBS buffer per 10^8 total cells. Cells were then ready for magnetic separation using Magnetic Separation (MS) columns and MACS separator magnet according to the number of labelled cells. An MS column was placed in the magnetic field and rinsed with 500 μl of PBS buffer. The cell suspension was applied onto the column and washed three times with 500 μl of PBS buffer. Unlabelled cells passed through the column during washing whereas labelled cells remained in the column. The column was then removed from the magnet and placed on a collection tube where the cells were flushed out by firmly applying the plunger supplied with the column using 1 ml of PBS buffer. To check the purity of the sorted cells (Fig 2.12), a small aliquot was further stained with 4 μl of PerCP anti CD3 antibodies (Becton Dickinson) and 4 μl of FITC anti CD8 antibodies (Becton Dickinson) in 100 μl of PBS for 20 to 30 min

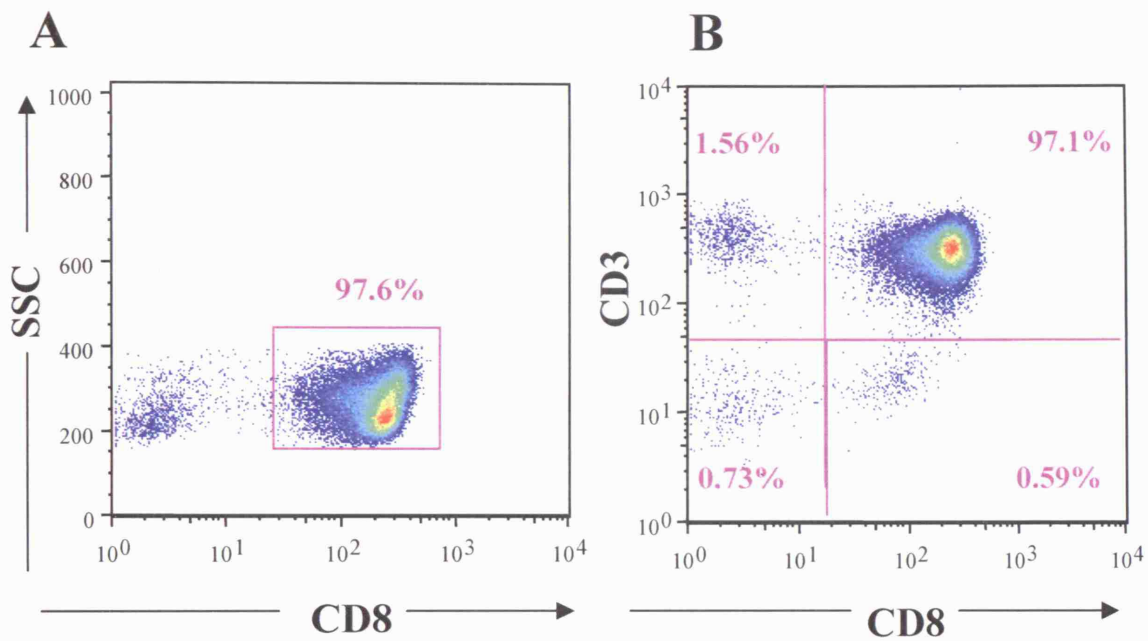


Figure 2.12.

CD8+ T CELL SELECTION: Purified CD8+ populations were isolated in order to determine the direct effect of the artificial APCs on T cells. Using anti CD8 magnetic micro beads, CD8+ T cells were isolated from the PBMC population with very high purity (~97%). **A.-** Shows purified cells gated on side scatter and CD8 staining and **B.-** Shows purified cells gated on CD8 and CD3 staining.

at 4°C in the dark. The cells were then washed twice and finally resuspended in 100 µl of PBS containing 1% paraformaldehyde (Sigma-Aldrich) buffer to fix the cells. Fixed samples in this manner were analysed straight away or they can be stored for up to one or two weeks before analysis using a FACS Calibur Flow Cytometer with Cell Quest acquisition software (Becton Dickinson) and Flow-Jo software.

2.5.4 aAPC *Ex-Vivo* Stimulations

At least 1×10^6 PBMCs or purified CD8 positive T cells (cultured in X-vivo medium containing 10% AB serum and 0.1% of penicillin/streptomycin all from BioWhittaker) from naïve (CMV negative) and/or previously exposed (CMV positive) individuals were stimulated with a specific batch of liposomes from the 3 ml stock solution of liposomes (Figure 2.13). Stimulations were performed every ten days in the presence of IL-7 (Rathmell, Farkash et al. 2001) (10 ng/ml). Tetramer staining, IFN- γ Intracellular staining and/or degranulation assays were performed three days after each stimulation after which, IL-2 (20 U/ml) and IL-15 (Alpdogan, Eng et al. 2005) (10 ng/ml) were also added. All cytokines were purchased from R&D Systems.

2.5.5 T Cell Antigen Specific Sorting

Tetramer positive cells can be specifically sorted in order to perform adoptive immunotherapy or simply to carry out functional and structural assays such as spectratyping, degranulation assays, cytokine profiles, etc. 1 µg of PE-A2-NLV tetramers were added to approximately 5×10^6 PBMCs following expansion with the aAPCs for 25 minutes at 37°C in 50 µl of PBS pH 7.2 buffer, supplemented with 0.5% BSA and 2 mM EDTA (4-8°C). Following washing, the cell pellet was resuspended in 80 µl of PBS buffer per 10^7 total cells and 20 µl of Anti-PE Micro-Beads per 10^7 total cells were added for 15 minutes at 4°C. Cells were washed again and resuspended in 500 µl of PBS buffer. Cells were then ready for magnetic separation using Magnetic Separation (MS) columns and MACS separator magnet



Figure 2.13.

FLUORESCENT IMMUNO LIPOSOMES: 15 batches of liposomes as aAPC can be generated in less than 48 hrs at the same time. Each batch contains a 3 ml volume and the liposomes from each batch can be coated with different amounts and/or ratios of MHC peptide complexes and costimulatory signals. Every batch can be different if required. These batches were already sterilized and ready to use. The liposomes shown are pink as a result of the rhodamine fluorescent lipid.

as previously described for the CD8⁺ T cell sorting. To check the purity of the sorted cells (Figure 2.14), a small aliquot was further stained with the A2/NLV relevant tetramer followed by 4 μ l of PerCP anti CD3 antibodies (Becton Dickinson) and 4 μ l of FITC anti CD8 antibodies (Becton Dickinson) in 100 μ l of PBS for 20 to 30 min at 4°C in the dark. The cells were then washed twice and finally resuspended in 100 μ l of PBS containing 1% paraformaldehyde (Sigma-Aldrich) buffer to fix the cells. Fixed samples were analysed using a FacsCalibur Flow Cytometer with Cell Quest (Becton Dickinson) and Flow-Jo software.

2.5.6 Dendritic Cell Cultures

In order to create a useful and eventually superior antigen presenting cell system, our artificial APC needed to be compared against the standard current approach, which is the use of dendritic cells for T cell activation and expansion. Furthermore as described later in chapter 4, the artificial APCs were found to interact directly with T cells but also semi directly via natural APCs. In order to compare and study these interactions, dendritic cultures were prepared. The generation of dendritic cells from peripheral blood was accomplished as follows: Following PBMCs separation, adherent cells were isolated by plating out 20 to 40 $\times 10^6$ PBMCs in 5 ml of RPMI 10% FCS into a single well of a 6 well plate for 2-4 hrs at 37°C. Non-adherent cells were then removed and frozen for future use as stimulator or responder cells. Seven days before the dendritic cells were to be used, the adherent cells were cultured in the presence of IL-4 (R&D Systems) at 100 ng/ml and GM-CSF (R&D Systems) at 250 ng/ml to promote the differentiation of monocytes into DCs for 2 days. Double the concentrations of IL-4 (200 ng/ml) and GM-CSF (500 ng/ml) were then added at day -5. One day before the dendritic cells were to be used (day -1), an aliquot of immature dendritic cells was taken from the well for use as an untreated control for FACS analysis (see next). Following this, 60 μ g/ml of Poly IC (Sigma-Aldrich) and 20 ng/ml of TNF α (R&D Systems) were added to induce full maturation of the DCs. Both Poly IC and TNF α trigger activation and maturation of DCs into highly effective antigen presenting cells. At day zero, FACS

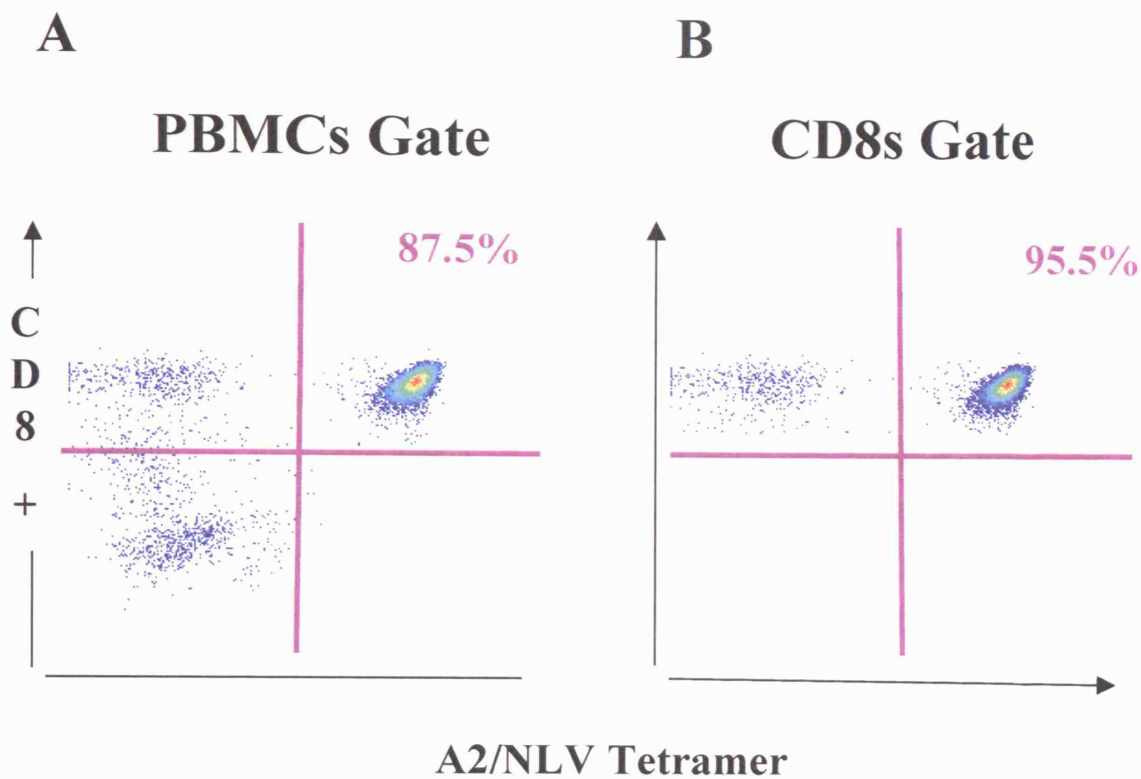


Figure 2.14.

T CELL ANTIGEN SPECIFIC SORTING: Tetramer positive cells can be specifically isolated before or after expansion in order to perform adoptive immunotherapy or to perform functional and structural assays. In this particular case, antigen specific CD8+ T cells were isolated following expansion using the coated immunoliposomes as aAPC using PE labelled tetramers and anti PE magnetic beads accomplishing high purity (~95%). **A.-** Shows CD8+ NLV tetrame positive cells isolated from PBMCs following aAPC stimulation (gated on PBMCs). **B.-** Shows CD8+ NLV tetramer positive cells isolated from PBMCs following aAPC stimulation (gated on CD8+ cells).

analysis was performed on Poly IC and TNF α treated and untreated cells to confirm DC maturation (figure 2.15). A cocktail of antibodies (all from Becton Dickinson) was used to assess the cultures (CD3, CD11c, CD14, CD19, CD56, CD80, CD83, CD86 and HLA-DR). CD3 negative staining confirmed that the final cells produced were not T cells, CD 56 negative staining confirmed that the cells were not NK cells and CD19 negative staining confirmed that the cells were not B cells. CD11c is a costimulatory molecule, whereas CD14 is a monocyte cell marker, CD80, CD83 and CD86 are all costimulatory markers and their up-regulation is essential to confirm DC maturation. Equally important is the high expression of class II molecules, which confirms DC maturation. FACS analysis gives a definitive confirmation of DC maturation. However, looking at the cells under the microscope is a simple way to double assess DC maturation since very large DC membrane prolongations (only seen on mature DCs) can be easily detected along and across the well plate (data not shown). Once DC maturation was established, DCs were harvested and pulsed for 2-4 hrs with either the A2 NLV peptide, irrelevant peptide or left unpulsed. Following this, DCs were concentrated to 0.3×10^6 /ml and used for different culture conditions. When T cells were stimulated with DCs, the cultures were set up at a DC: CD8⁺ ratio of 1:10 plus the irradiated negative fraction (25 Gy for 7 minutes) to provide CD4 help. IL-7 (10 ng/ml) was also added at the time of the first stimulation and IL-2 at 20 U/ml was added at day 7. At day 12, peptide pulsed irradiated PBMCs containing IL-2 were used to re-stimulate the cultures at a ratio of 5:1. DCs can certainly be used to re-stimulate the cultures (Subklewe, Chahroudi et al. 1999), however this can lead to excessive over stimulation and the cultures die in a short space of time. Therefore T cell cultures were re-stimulated with peptide pulsed PBMCs as described by Mutis (Mutis, Verdijk et al. 1999).

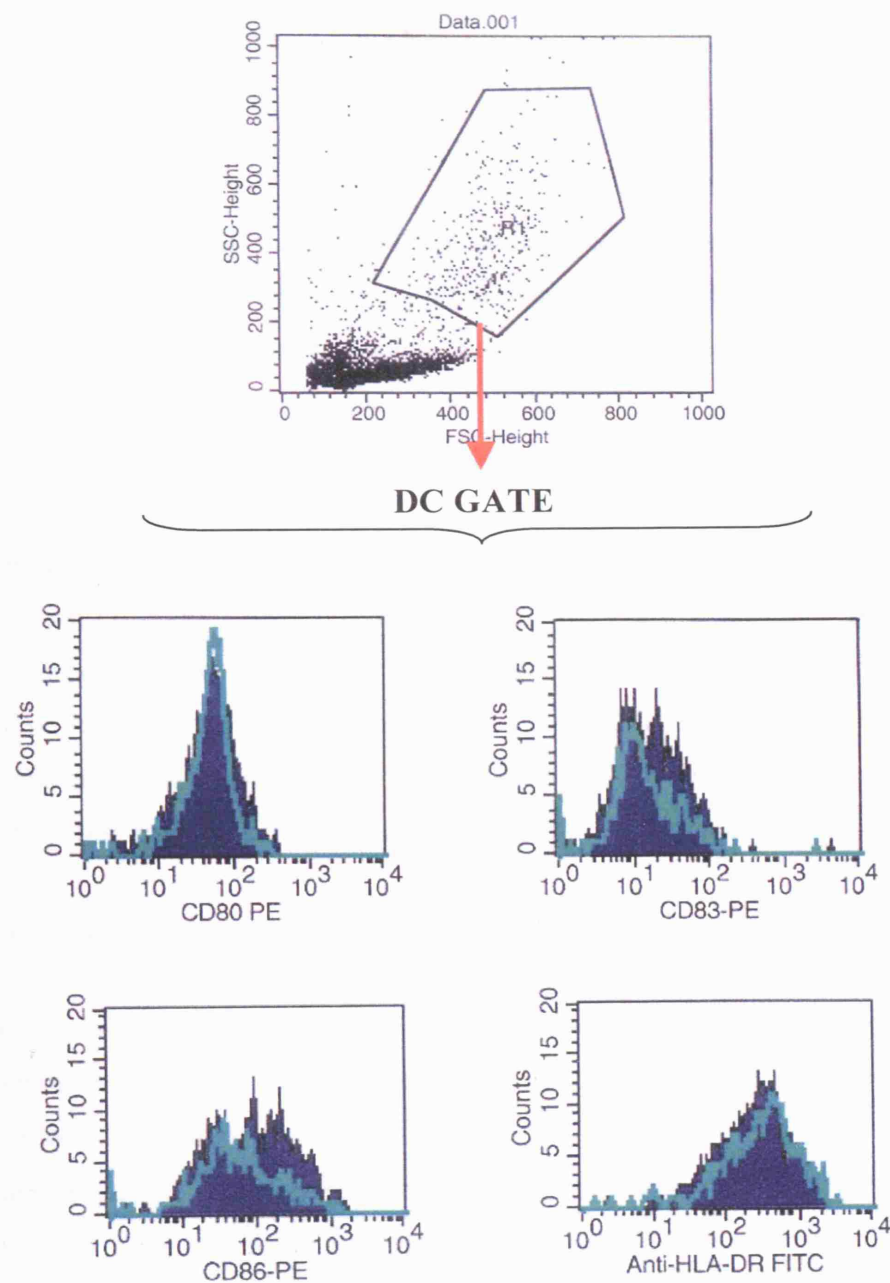


Figure 2.15.

DC MATURATION: A cocktail of antibodies was used to assess DC maturation. Up-regulation of CD80, CD83 and CD86 on mature DCs (dark blue) compared against immature DCs (in green) are amongst the most important markers as well as a high expression of class II molecules (anti HLA-DR). Immature and mature DCs were compared in parallel in order to ensure DC maturation.

2.6 FUNCTIONAL ASSAYS

2.6.1 T2 Cells

TAP deficient cells such as T2 cells (used as targets in our assays), fail to express MHC cytosolic peptide complexes on their surface since peptide transport from the cytosol to the endoplasmic reticulum (ER) is blocked. However T2 cells can form and present MHC peptide complexes by loading self-peptides within the ER. These peptides however are rather weak binders and even though they reach the cell surface, they are not sufficiently strong to maintain stable complexes at the surface and they are quickly recycled. We take advantage of this feature by feeding T2 cells with a strong known peptide binder such as the NLV pp65 CMV peptide, these peptides compete with the weak self peptides presented at the T2 cell surface and stabilise the MHC molecules at the surface (Barber, Whitelegg et al. 2004).

T2 cells are easily maintained in culture for long periods and therefore are a great source of targets in functional assays.

2.6.2 Tetramer Staining And Flow Cytometry

A minimum of 10^5 PBMCs or T cells were plated in a 96 “v” well plate (Nunc, Denmark) and washed by spinning for 3 min at 1700 rpm/4°C and the supernatant discarded. The cells were first stained with 1 µl of PE conjugated relevant or irrelevant tetramer and resuspended in 100 µl of sterile PBS buffer for 30 min at 37°C. Following washing for 3 min at 1700 rpm/4°C, 4 µl of PerCP anti CD3 antibodies and 4 µl of FITC anti CD8 antibodies (both from Becton Dickinson) were added to the cells in 100 µl of PBS for 30 min at 4°C in the dark. The cells were then washed twice and finally 100 µl of PBS containing 1% paraformaldehyde (Sigma-Aldrich) buffer were added to fix the cells. Fixed samples were analysed using a FACS Calibur Flow Cytometer with Cell Quest (Becton Dickinson) and FlowJo software.

2.6.3 Phenotypic T Cell Characterization

In order to determine both the general and antigen specific T Cell Phenotype, following tetramer staining as described above, 4 μ l of FITC anti CD27, APC anti CD45RO and PerCP anti CD8 antibodies (all from Becton Dickinson) were added to the cells in 100 μ l of PBS for 30 min at 4°C in the dark. The cells were then washed twice and finally 100 μ l of PBS containing 1% paraformaldehyde (Sigma-Aldrich) buffer were added to fix the cells. Fixed samples were analysed using a FacsCalibur Flow Cytometer with Cell Quest (Becton Dickinson) and Flow-Jo software.

2.6.4 IFN- γ Intracellular Staining

As previously mentioned in the introduction, in addition to cytotoxicity, T cells also secrete cytokines upon antigen recognition and TCR engagement. In the case of CD8⁺ T cells, IFN- γ is one of the more important cytokines. Among its functions, IFN- γ induces up-regulation of MHC class I molecules on target cells making them "more visible" to T cells and more likely to be killed. INF- γ also recruits APCs to the site of infection in order to amplify the immune response if needed. One of the most important features of INF- γ is that it is normally synthesized *de novo* upon antigen recognition by T cells. Therefore T cell INF- γ detection is often used as a way to measure T cell functionality in anti viral T cell responses. IFN- γ production can be measured in the stimulated general T cell population, but also in particular and specific populations such as those that are tetramer positive. For its detection, a minimum of 10^5 cells were washed for 3 min at 1700/4°C. 1 μ l of relevant tetramer was added for 30 min at 37°C in 50 μ l of sterile PBS buffer. Cells were then washed to remove uncoupled tetramer and stimulator cells added (10 μ g/ml pp65 CMV peptide pulsed or non-pulsed autologous PBMCs at a 2:1 responder/stimulator ratio) to trigger IFN- γ production, which peaks between 4-5 hrs after stimulation. Brefeldin A (Sigma-Aldrich) was added after an hour (10 μ g per well) for a period of 5 hours. This prevents IFN- γ release in order for it to be retained intracellularly

so that it can be detected inside the cells. Cells were then washed and 2 µl of APC anti CD8 and PerCP anti CD3 antibodies were added for 30 minutes at 4°C in the dark. Cells were washed twice and resuspended in 100 µl of cytofix/cytoperm solution (PharMingen) for 20 min at 4°C in order to make the cells permeable. Cells were washed twice using cytofix/cytoperm washing buffer (PharMingen) and then resuspended in 50 µl of cytofix/cytoperm buffer containing 2 µl of either PE anti IFN-γ antibody or PE isotype control antibody (both from PharMingen) for 20 min at 4°C in the dark. Finally cells were washed twice and resuspended in 100 µl of 1% paraformaldehyde fixing buffer. Cells were then analysed by Flow cytometry as shown later in chapter 4.

2.6.5 Degranulation Assay

Since T cell proliferation and expansion does not always correlate with functional response (Monsurro, Nagorsen et al. 2002), one of the concerns related to T cell expansion by dendritic cells or artificial antigen presenting cells is the cytolytic potential of the expanded T cells. To assess the cytolytic potential of the expanded tetramer positive cells by the artificial APCs, CD107 mobilization assays (BD Pharmingen) combined with tetramer staining were performed. For the assay, either irrelevant and CMV-NLV peptide pulsed T2 cell targets and stimulator cells were co-cultured in a 2:1, 1:1 and 1:2 ratio at 37°C for 5 hrs in the presence of 2 µl of FITC anti CD107 a and b antibodies respectively. The expression of CD107 proteins at the cell surface can be observed as early as 30 min following stimulation and reaches a maximum at 4 hrs of stimulation. Their expression therefore indicates T cell degranulation. At the 1st hr of culture, 1 µl of 2 mM Monesin (BD Biosciences) was added to the cultures. After 5 hrs, tetramer staining and IFN-γ intracellular staining were also performed as described previously. The cells were then fixed and analysed by Flow cytometry. Even though the CD107 assay does not directly measure target cell lysis, it does provide a measure of the cytotoxic potential and killing properties of the T cells of interest, in this case the artificially expanded antigen specific CD8⁺ T cells. Furthermore by combining the CD107

assay with tetramer staining, intracellular staining and phenotypic T cell characterization, the functionality of the T cells generated by the artificial APCs was assessed in greater detail (Wolint, Betts et al. 2004) as discussed in chapter 4.

2.7 *IN VIVO* MOUSE MODEL

Following animal house regulations (Animal Scientific Procedures Act 1986), Balb/c mice (figure 2.16) were injected at Nottingham Trent University in duplicates with 200 µl of different fluorescent liposomes in order to evaluate their *in vivo* presence. Coated and uncoated liposomes as well as super para magnetic iron oxide nano particles (spios) loaded and unloaded liposomes (as explained in chapter 3 and 5) were injected intravenously (i.v.) via the tail vein. Previous to injection, mice were given anaesthetic cream (Astra Pharmaceuticals) at the site of injection and put into 37°C chambers to induce vasodilatation and facilitate the injection. Mice were sacrificed at different time points: 2 hrs, 24 hrs, 48 hrs and 72 hrs after injection. Blood and organs were harvested, cryopreserved, sectioned and analysed for liposome detection under fluorescent microscopy whereas for whole body imaging, other mice were analysed by magnetic resonance imaging (MRI) as explained in chapter 5.

2.8 MISCELLANEOUS

2.8.1 Desalting Columns

PD-10 desalting columns from GE Healthcare, are prepacked and disposable columns containing Sephadex G-25 medium for group separation of high from low molecular weight substances by desalting and buffer exchange. Separation protocol: to prepare the column, the bottom tip is cut off and the top cap removed. The column is equilibrated with 25 ml of desired buffer. The sample at a total volume of 2.5 ml is added discarding any flow-through coming from the bottom of the

column. Elution and recovery of the interest sample is collected by passing 3.5 ml of buffer accomplishing purity typically greater than 95%.

2.8.2 MRI Equipment

Magnetic Resonance Imaging was performed in collaboration with Dr. Martin Bencsik at Nottingham Trent University. The MRI equipment used for the studies was a vertical (Magnex Scientific Ltd) superconductive magnet manufactured by Bruker that accommodates samples up to 30 mm in diameter (figure 2.17) and works at 9.7 Teslas.



Figure 2.16.

IN-VIVO MOUSE MODEL: Balb/c mice were used in order to determine the kinetics, behavior and MRI detection of the artificial APCs.

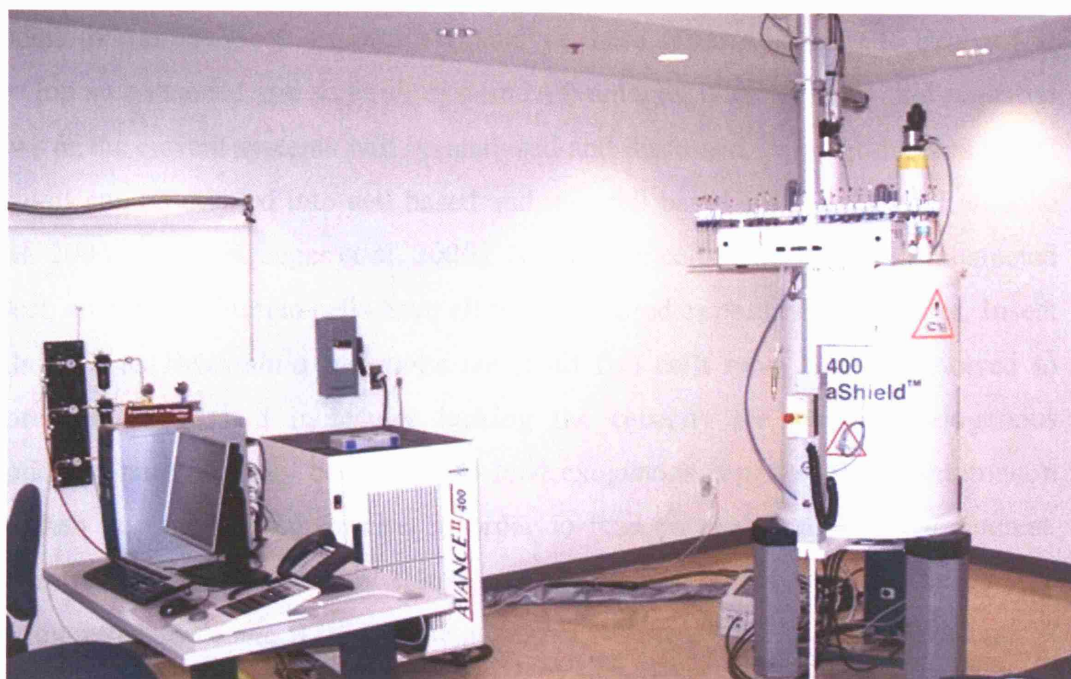


Figure 2.17.

MRI EQUIPMENT FOR ANIMAL STUDIES: Balb/c mice were MRI scanned using this machine. The mouse is placed vertically inside the white cylinder magnet during the study, which takes between five to ten minutes depending on the level of resolution desired.

CHAPTER 3

CONSTRUCTION OF A SUPER PARA MAGNETIC ARTIFICIAL NANO APC FOR ACTIVE AND ADOPTIVE IMMUNOTHERAPY

3.1 INTRODUCTION

As discussed in the general introduction, many of the factors of the current artificial antigen presenting cell (aAPC) systems such as potency, speed of expansion, quality of the T cells generated, specificity and biosafety, vary among different models. It was therefore important to analyse these systems in order to evolve and develop an enhanced and superior system. Advantages, disadvantages and potential flaws of the current systems will be analysed and discussed. In general terms aAPC systems can be divided into cell based and non-cell based systems (Kim, Latouche et al. 2004; Oelke, Krueger et al. 2005). Among the cell-based systems, transfected insect, animal and human cells have all been explored as possible candidates. Insect cells such as *Drosophila melanogaster* (fruit fly) cells have been engineered to express MHC class I molecules lacking the capacity for loading endogenous peptides. Therefore only being able to load exogenous peptides, this phenomenon can then be manipulated *ex vivo* in order to load particular peptides of interest. These cells have also been transfected with the cDNA sequences for the costimulatory molecule B7.1 and the adhesion molecule ICAM1 (Cai, Brunmark et al. 1996). Despite this, the system was not able to stimulate purified CD8⁺ populations; natural APCs and feeder cells were required in the cultures in order to induce T cell stimulation. This failure in CD8⁺ T cell stimulation was partially explained by the lack of both CD4 help and cytokines produced by other cells, which are known to play a critical role during antigen presentation. However with this system, even in the presence of natural APCs and CD4 T cells, it is difficult to state that the transfected insect cells were truly working as artificial APCs because

insect cells are unstable at 37°C, leading to their rapid destruction and to a massive release of *Drosophila* antigens. These released antigens could function as danger signals inducing the natural APCs to mature. Simultaneously these natural APCs could take up the peptides released by the destroyed insect cells and cross present peptides using both self MHC molecules and co-activatory signals. This could explain the T cell expansions observed using this system. Furthermore the instability of insect cells at 37°C also importantly limits the time of interaction between T cells and artificial APCs, which masquerades their true mechanism of action, factors that enormously limit their application. Therefore more stable systems have been explored. Among these, transfected animal cells such as mouse fibroblasts have been retrovirally transfected with six genes encoding the respective sequences for ICAM1 and LFA-3 as adhesion molecules, B7.1 as costimulatory signal, an HLA A*0201 class I molecule, β_2m and peptides of interest i.e., CMV pp65 or WT1 (Latouche and Sadelain 2000). This system has proven to be as effective as the use of DCs as APCs and in use was able to expand purified CD8⁺ T cells to achieve 30% of tetramer positivity for the relevant peptide in less than two weeks after a single stimulation. This system was generated to expand T cells *ex vivo* for subsequent therapeutic infusion as an adoptive immunotherapy strategy (Papanicolaou, Latouche et al. 2003). The system however, could not be used either *ex vivo* or *in vivo* for a number of reasons: Firstly, a strong xenoreactive response is mounted against mouse antigens leading to the rapid clearance of the mouse cells, which might not be as rapid in immunocompromised patients depending primarily on their number of T cells. However potential tumorigenicity could emerge by accidental *in vivo* injection since gene manipulation and transfection has been performed on these cells, a potential although unlikely engraftment of the cells in the new host could exist plus the potential unknown hazards of introducing murine DNA into humans. Despite such problems, cell based systems have provided a great understanding of the adhesion and costimulatory signals required to expand antigen specific CD8⁺ T cells *ex vivo*. Other murine models have also been explored such as the murine plasmacytoma cell line J558L. This has been transfected to express

MHC-Ig Dimers (Schneck 2000), which share some of the advantages but also disadvantages of the mouse fibroblast system previously described.

Transfected human cell lines such as K32 cells have also been explored as aAPCs. K32 cells are derived from a K562 precursor cell, which is a chronic erythroleukemic cell line negative for HLA-A, B and DR molecules but positive for HLA-C, ICAM1, B7.1 and LFA-3 molecules. K32 cells have been transfected with CD32 (Maus, Thomas et al. 2002; Thomas, Maus et al. 2002), which is the receptor for the FC region of IgG antibodies. This transfection allowed K32 cells to be coated with antibodies against CD3 and CD28 receptors. Using this system the authors were able to demonstrate a 1000 fold increase of polyclonal CD4⁺ T cells in 30 days, but not CD8⁺ T cells. CD8⁺ T cells were not expanded by the use of this system because of the lack of 4-1BB expression on the surface of the K32 cells (Maus, Thomas et al. 2002). This study showed that even though K32 cells express HLA-C molecules that could have induced polyclonal or antigen specific expansion of CD8⁺ T cells to an extent, the lack of 4-1BB expression avoided such expansion, which was reverted by transfecting the K32 cells with the 4-1BB receptor sequence. This particular result provided confirmation of the importance of 4-1BB for CD8⁺ T cell expansion. Therefore its use in artificial APC systems aiming to stimulate CD8⁺ T cells should be mandatory. It is important to highlight that the expansion of the CD4⁺ T cells generated by the use of K32 cells is not antigen specific and this is due to the lack of DR class II molecules on the surface of the K32 cells, a property that could be eventually accomplished by coating the cells with MHC class II/peptide complexes. However adoptively infused CD4⁺ polyclonal *ex vivo* expanded T cells, could provide a useful therapeutic effect in HIV patients for instance, who are in need of CD4⁺ polyclonal cells. Unfortunately this system is unsuitable for both *ex vivo* and *in vivo* applications since the system is based on an immortalised leukaemic cell line that has been further transformed. For adoptive immunotherapy, the risk of infusing both K32 cells and expanded T cells could potentially generate a B cell lymphoma or leukaemia in the patients. For the same reasons, their use *in vivo* for active immunotherapy has never been considered. This

system however has given valuable information about signal combinations required to expand different subsets of T cells such as the use of 4-1BB for the CD8 T cells. Some of the most relevant advantages and disadvantages concerning insect, animal and human cell based systems for use as artificial APCs have been described. However probably one of the most important criticisms concerning all cell based systems, is that they, as well as natural APCs and other human cells, are susceptible to factors responsible for human disease as they are prime targets of immune evasion and suppression mechanisms as manifested by tumours and pathogens such as immunosuppressive cytokines and viral infection, which induce, depending on the virus, MHC class I and II down regulation as previously discussed in the introduction. Such problems initiated the investigation and potential application of non-cell based aAPCs. An example of such a system would be the use of magnetic beads covalently linked to different monoclonal antibodies (mAbs). This system is currently in use as an aAPC for T cell expansion. These beads are mainly coated with anti CD3 and anti CD28 mAbs. However as seen with K32 cells, only CD4⁺ T cells are expanded in a polyclonal manner (Lum, LeFever et al. 2001). In order to elicit antigen specificity, beads have been coated with mAb against either MHC class I or class II molecules together with the anti CD28 mAb (Maus, Riley et al. 2003; Oelke, Maus et al. 2003; Schilbach, Kerst et al. 2005). In these experiments, a CMV peptide was used as model for the class I approach and an influenza matrix peptide for the class II approach. CD8⁺ CMV peptide and CD4⁺ influenza matrix peptide tetramer positive T cells were sorted out from the bulk of the cells and then stimulated accordingly with either the class I or class II magnetic beads also coated with anti CD28. Using these stimulation protocols, expansions of 7 fold have been reported after one round of stimulation. This level of expansion even though relevant, is not particularly high and could be partially explained by the lack of 4-1BB in this system, which is critically important for the CD8⁺ T cells, and OX40 ligand which is equally important for the CD4⁺ T cells. Although not usable for infusion *in vivo*, magnetic beads could be considered as tools for the *ex vivo* expansion of T cells. The limitations of this system are its rigidity, the potential need to detach the beads from the expanded T cells to prevent micro embolisms and

the immunogenicity of the linked mAbs. Nevertheless magnetic beads have been useful in demonstrating that cross linking between MHC peptide complexes and one or two costimulatory signals are sufficient to accomplish T cell antigen specific expansion.

Another relevant non-cell based system with more potential is the use of exosomes. As mentioned previously, exosomes have a 30-100 nm diameter and are vesicles, which can be derived not only from dendritic cells, but also from tumour cells and potentially all other cell types (Thery, Zitvogel et al. 2002). As an artificial APC system, exosomes have been used in a number of ways. Either patient or third party dendritic cells are cultured *ex vivo* by standard protocols, the culture supernatants containing secreted exosomes are recovered, concentrated and purified. Dendritic cell derived exosomes contain MHC class I and II/peptide complexes, adhesion (ICAM1) and costimulatory (B7.1) signals (Segura, Amigorena et al. 2005; Segura, Nicco et al. 2005). In order to have dendritic cell derived exosomes expressing the MHC/peptide complexes of interest, DCs are peptide pulsed during culture, i.e., indirect loading. In doing so, many of the exosomes will express the desired complexes i.e., HLA-A2 restricted MART-1 tumour peptide complexes (Hsu, Paz et al. 2003). However, the peptide of interest can also be loaded more efficiently after exosome purification, i.e., direct loading. Once recovered from culture, exosomes are exposed to an acidic buffer (4.2 pH) in order to denature their MHC/peptide complexes. β_2m and the peptide of interest (MART-1) are then added, the sample is re-equilibrated to pH 7 and the newly refolded MHC/peptide molecules on the exosome surface will contain the desired peptide epitope. A modification of this has been reported by the same group where at pH 5.2, only partial MHC-peptide complex unfolding occurs. This only requires the exchange of peptides thus avoiding the use of β_2m for the new refolding. Direct loading allows the generation of higher numbers of desired MHC/peptide complexes than indirect loading. Any unbound peptide by whichever mechanism is then removed by filtration and the exosomes are ready for use. The exosomes directly or indirectly loaded bearing the desired MHC peptide complexes (HLA-A2/MART-1) are then used to prime immune responses from naïve individuals *in vitro* (Andre, Chaput et

al. 2004). Interestingly however, exosomes themselves cannot prime or expand T cells directly since they do not function as micro APCs (Wolfers, Lozier et al. 2001; Thery, Duban et al. 2002; Andre, Chaput et al. 2004). In order to function correctly, exosomes require dendritic cells present in the culture. An investigation of their mechanism of action has shown that exosomes are incorporated and acquired by the membrane of natural APCs, whether exosomes are incorporated onto the membrane or are fused into is not exactly known. However dendritic cells acquired exosomes allow the DCs to reach a state of maturation enabling them to mount specific immune responses without the need for antigen processing themselves (Thery, Zitvogel et al. 2002). Cross presentation by DCs of the peptides delivered on the surface of the exosomes as an alternate mechanism of action could potentially take place and needs to be investigated. However confirmation of exosome function at the cell surface has been confirmed by an elegant study where allo MHC peptide complexes on the surface of exosomes produced by dendritic cells from a third party were found intact on the surface of dendritic cells from patients undergoing solid organ transplantation. These allo MHC peptide complexes acquired by host DCs via exosomes were able to induce strong allograft responses (Herrera, Golshayan et al. 2004). These results confirmed that exosomes function primarily at the cell surface of the natural APCs. Initial results using exosomes as adjuvants in mounting immune responses look promising as they can be used *in vivo* (Lamparski, Metha-Damani et al. 2002; Morse, Garst et al. 2005). In the latter phase I clinical trial carried out by Morse et al in the US, dendritic cell derived exosomes were recovered from patients with advanced non-small cell lung carcinoma. The exosomes were loaded with MAGE peptide from the lung carcinoma and were given back to the patients. Nine patients completed the therapy and functional anti MAGE-specific T cell responses were detected in 3 of them experiencing long term stability of the disease. This study demonstrated the feasibility of this form of therapy, which is attractive since the exosomes can be loaded with peptides of choice. Unfortunately, in order to recover sufficient amounts of exosomes from DCs, a large volume of blood from the patient is required to obtain a sufficient number of dendritic cells. This is not always possible

depending on the general condition of the patient and blood from several donors may be required. Another drawback is that the dendritic cells need to be cultured with the attendant disadvantages that this has such as the high cost of the process due to the high concentration of cytokines needed in the cocktails required for DC expansion, the time consuming nature of the protocol, the lack of reproducibility among samples and the necessary lengthy manipulation of DCs themselves which is not ideal. These drawbacks currently make the potential use of exosomes uncertain. Other simple systems have also provided important information in the field of artificial antigen presentation such as tetramers, modified tetramers and multimers. Tetramers consist of four-biotinylated MHC class I or II / peptide complexes (monomers) linked to a streptavidin molecule. The use of tetramers as a detection tool for antigen specific T cells is widely used and accepted. This system for antigen specific T cell detection has also become a means of antigen specific T cell stimulation. Soluble class I tetramers have been administered *in vivo* and were shown to prime specifically naïve T cells against the Hy minor antigen in a mouse model (Maile, Wang et al. 2001). Surprisingly, T cell priming was accomplished without the need for any co-activatory signals, which is strongly suggestive of cross presentation of the peptide present on the tetramers and delivery by natural APCs. These primed T cells in females were shown to be functional by rejecting male skin grafts. Although these studies showed encouraging *in vivo* results, it is well described that without co-activation, MHC/peptide engagement itself on naïve T cells produces tolerance by deletion or anergy either in the thymus or the lymph nodes (Fairchild and Austyn 1990; Hawiger, Inaba et al. 2001; Steinman, Hawiger et al. 2003), which suggests that tetramers are cross-presented by DCs or other cell *in vivo* interactions are taking place as the mechanism of action. The important point to emphasise is that on T cell activation, co-activatory molecules are necessary to accomplish priming of naïve T cells and full T cell activation of memory cells. This severely limits the use of tetramers as artificial APCs. It is now possible however, to substitute one of the arms of a tetramer to present 3 monomers and one costimulatory molecule in the form of biotinylated mAbs such as anti CD28 or anti CD27. As with other systems, these modified tetramers can stimulate

CD8⁺ T cells and the conditions required for this are under investigation here at the Anthony Nolan Research Institute. Their *in vivo* use is unlikely because their co-activatory capacity is limited and an immune response will also be generated against streptavidin and the FC regions of the mAbs. However their *ex vivo* potential is promising as large batches of long lasting modified tetramers can be generated with different co-activatory signal combinations. Recently the generation of multimers attached to carbohydrate chains have also been explored as aAPCs to generate specific CD8⁺ T cell expansions. This system can carry multiple co-activatory molecules. However their *in vivo* use is uncertain as the risk of micro embolisms are possible if T cells do not detach themselves from the multimer chains plus the disadvantages previously stated for modified tetramers such as the immunogenicity of the FC regions of the antibodies. However their *ex vivo* application as aAPC is also promising as large long lasting batches can be produced and several co-activatory signals can be presented simultaneously.

A simple way of stimulating antigen specific CD8⁺ T cells *ex vivo* is the addition of class I tetramers and soluble non super agonist antibodies as co-activatory molecules for CD28, CD27 and CD40 ligand among others (Dr. Sylvie Rusakiewicz, personal communication). Due to the equal distribution of these molecules in cell culture, antigen specific CD8⁺ T cells are expanded to an extent. The problem with this type of stimulation is that the phenotype of all the expanded cells is terminal effector. As a consequence the cells are committed to an effector function, which is immediately followed by induced cell death or apoptosis. Therefore these cells have a short life expectancy. This is a disadvantage since the final goal of artificial antigen presentation by artificial APC systems is not only to by-pass compromised antigen presentation or DC malfunction, but also the generation of effector and long lasting memory immune responses in order to deliver a strong clinical effect in patients.

All systems discussed generate expansions with different kinetics; factors such as lytic activity, survival potential and homing pattern of the expanded T cells according to the phenotype generated with each system are also different and remain to be published. Ideally systems that are optimised for the generation of both

effector and memory T cells are the main objective in order to deliver cells with cytotoxic properties in the short term, and also cells to maintain immunological memory in the long term. Although the field of artificial antigen presentation is growing and improving, safety and clinical usage is also a priority. Due to the many complications of the current aAPC systems when used *in vivo* such as the obvious safety reasons concerning the use of live cells from non-human sources, make these systems very difficult to translate into clinical settings. This together with fears of micro embolisms, the immune responses against streptavidin used in modified tetramers and the response generated against the FC regions of the mAbs used in almost all the systems, has basically restricted the use of these models as artificial APCs for *ex vivo* T cell expansion. Dendritic cell derived exosomes can be used *in vivo*, however as previously mentioned, their usage as semi-direct aAPCs remains unclear due to the extensive, difficult and long process needed to obtain them. Although tetramers and modified tetramers could also be used *in vivo*, the lack of costimulation with tetramers, the limited delivery potential of modified tetramers and the immunogenicity of streptavidin and the FC antibody regions, limits their *in vivo* use considerably.

Thus far a comprehensive discussion concerning the advantages and disadvantages of many of the current and popular artificial APC systems has been outlined. This information was very helpful to plan the strategy for the construction of a novel artificial APC envisaged for both *ex vivo* and *in vivo* clinical application. Therefore important factors concerning adoptive and active immunotherapy were also key to be considered. Adoptive immunotherapy or the *ex vivo* expansion of T cells for subsequent therapeutic infusion is expanding since as highlighted in detail in chapter 4, it can rescue and cure patients with malignant diseases and otherwise fatal viral infections. However one of the main concerns with adoptive immunotherapy is that it relies on one critical factor: *ex vivo* cell manipulation. The problems here are that the longer the *in vitro* T cell culture, the shorter the *in vivo* T cell survival after infusion (Dudley and Rosenberg 2003). *In vivo* artificial expansion systems for active immunotherapy would clearly circumvent this

problem. Nevertheless active immunotherapy is not always the ideal solution since in some patients, the T cells that could be potentially expanded *in vivo* are no longer present as they have been deleted, killed or anergised as a result of immune evasion and suppression strategies by tumours and pathogens. Therefore ideally a flexible system would be the best solution allowing one to performed both adoptive and active immunotherapy depending on the patient's requirements. Currently there is no comprehensive artificial antigen presenting cell system for both effective *ex vivo* and *in vivo* antigen specific T cell expansion. Therefore the construction of an artificial APC system posed challenging objectives regarding all the points previously discussed. It first needed to be a flexible system capable of being used both *ex vivo* and *in vivo* with the capability of trafficking in the body in a safe manner, biodegradable, non-toxic, non-tumourigenic, capable of forming immunological synapses and delivering signals for antigen specific T cell engagement, activation, expansion and survival. Furthermore according to nanotechnology directives as previously described (NIH 2005), it needed to be as small as possible in the 100 nm scale, which translates to structures 50 times smaller than cells, and yet traceable *in vivo*. Its size therefore suggested that it could not be a cell-based strategy. Cells are also susceptible to immunosuppressive cytokines, viral infection and MHC class I and II down regulation, which are very often responsible for either the disease itself or the malfunction of the original natural APCs in the first instance. A non-cell based strategy would normally facilitate an "off the shelf" availability but still, it also needed to be biodegradable and safe for *in vivo* use. Liposomes were chosen as vector and basic structure of the aAPC since they have been used *in vivo* in human clinical trials previously (1995; James 1995) and for the knowledge from human and animal studies regarding their safety, biodegradability and their lack of tumourogenicity (Vingerhoeds, Storm et al. 1994; Park, Hong et al. 1997). Therefore liposomes and more precisely targeted liposomes known as immunoliposomes were chosen to develop the artificial APC.

3.2 IMMUNOLIPOSOMES

Liposomes are spherical vesicles formed from bilipid membranes with liquid interiors that can be manufactured to a range of nano sizes and dependent on the particular intended purpose, can be generated from different lipid combinations. Since liposomes possess bilipid membranes, they can form immunological synapses with cells mimicking natural cell interactions (Prakken, Wauben et al. 2000; Giannoni, Barnett et al. 2005). They have therefore served as one of the main models for biological membranes. More recently nanomedicines based on liposomes have been developed: DoxilTM (doxorubicin in liposomes) is an anticancer drug and the first liposome delivered drug that was approved by the US Food and Drug Administration (FDA) in November 1995 to treat progressive or recurred ovarian cancer and AIDS related Kaposi's sarcoma. Encapsulation of drugs in liposomes was and is still increasingly exploited since drugs encapsulated in liposomes target to tumour sites more efficiently than the free drug. Although liposomes traffic through the body in an unrestricted fashion to brain, skin and internal organs, they target tumours more efficiently due to their Enhanced Permeability and Retention tumour effect (EPR) (Fang, Sawa et al. 2003). The EPR effect is explained by an intrinsic feature of tumours: because of their accelerated metabolic rate due to a fast cycle of cell division, tumours require large amounts of oxygen and nutrients. To fulfil these demands, they possess large numbers of blood vessels that are constantly dilated. The EPR effect sequesters the liposomes at the tumour site. Therefore liposomes traffic to tumours and remain there; this effect is not seen with free drugs. The EPR effect has revolutionised the use of liposomes for *in-vivo* applications. Studies have shown that the drug must be released from the liposomes into the tumour intercellular spaces in order to exert its anti tumour effect since liposomes themselves do not enter the tumour cells. The drug leaks out from the liposomes and the drug release rate, which takes several days, depends on the liposome membrane composition. One of the most recent applications is the use of adenoviral vectors for gene therapy entrapped in liposomes (Ma, Mi et al. 2002; Yotnda, Davis et al. 2004; Steel, Cavanagh et al. 2005). In doing this, the

adenoviral vector behaves as a liposome and not as a virus. Since the liposomes target the tumours as a consequence of the EPR effect, the adenoviral vector is only released at the tumour site producing delivery to tumour cells almost exclusively, no liver or spleen infection is observed, which has been fatal in patients in the past, and no immune responses against the vector are produced. DNA, RNA and plasmids have also been entrapped in liposomes and specifically targeted to tumours. In an effort to improve the effectiveness of liposomal formulations further, liposomes have been recently made targeted by coating them with antibodies or Fab regions against tumour antigens. Delivery has been improved and has been made even more specific.

Coated targeted liposome aAPCs could theoretically target tumours directly and T cell activation and expansion of memory cells could take place *in situ*. However coated liposome aAPCs should also traffic to lymph nodes producing T cell activation of naïve cells in a similar manner as natural APCs.

Liposome construction has been detailed in chapter 2. In brief, a mixture of five lipids was used: Phosphatidylcholine (PC); Cholesterol (Chol); 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N- [Methoxy (Polyethylene glycol)-2000] (DSPE-PEG); 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine -N- [Maleimide (Polyethylene glycol)-2000] (DSPE-PEG-MAL) and [1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine -N- (Lissamine Rhodamine B Sulfonyl)] DPE-RHODAMINE.

PC and CHOL give liposomes their basic core structure. PEGylated lipids (black tails in figure 3.1) are used to reduce/avoid recognition by the reticulo-endothelial system (Iden and Allen 2001; Maruyama 2002), allowing the liposomes to circulate in the body for several days. Pegylated lipids also facilitate 60% less uptake by macrophages, which are one of the main liposome clearance mechanisms. The MAL (maleimide) lipid represented as the yellow tail in the same figure allows binding of proteins such as MHC class I monomers, mAbs and Fab regions to the surface of the liposomes (see section 3.3). The rhodamine lipid allows the production of fluorescent traceable liposomes as explained in section 3.4.1 and DS lipids endow more stability to the liposome membrane in order for them to remain

stable in culture and in vivo, allowing for the study of the role of the APC in the immune response.

3.3 COATING LIPID-BASED VESICLES WITH MHC CLASS I MONOMERS, DIMERS AND PEPTIDE COMPLEXES

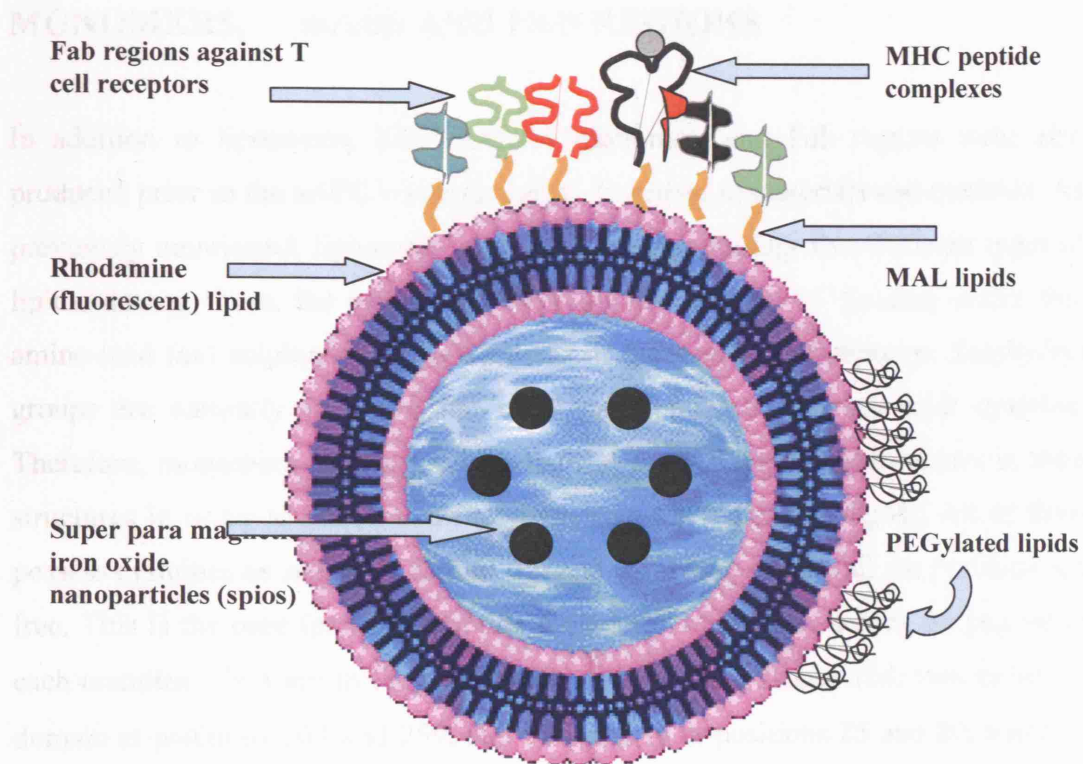


Figure 3.1.

ARTIFICIAL APC MODEL: 100 nm immuno-magneto-liposomes were generated following nanotechnology requirements. Among these, a double traceable APC was generated by the incorporation of a fluorescent (rhodamine) lipid in the constructs plus the loading of magnetic resonance imaging (MRI) traceable spios in the inner compartment (magnetoliposomes). On the surface, via maleimide (MAL) lipids, MHC peptide complexes, antibodies and/or Fab regions against T cell receptors were incorporated to provide immunological functionality (immunoliposomes): anti LFA-1 as an adhesion molecule, anti CD28 and anti CD27 as early activation signals, anti CD40L to attract CD4 help and also as a survival signal and anti 4-1BB as late activation signal. PEGylated lipids ensure long circulating *in-vivo* times by reducing rapid uptake by the reticulo endothelial system.

stable in cultures and *in vivo*. Liposomes, which are targetable are termed immunoliposomes.

3.3 COATING LIPOSOMES WITH MHC CLASS I MONOMERS, mABS AND FAB REGIONS

In addition to liposomes, MHC class I monomers and Fab regions were also produced prior to the aAPC construction as described in materials and methods. As previously mentioned, liposomes were generated by mixing five different types of lipids, among these, the maleimide lipid has the property of binding active free amino-acid (aa) sulphhydryl groups present in the structure of proteins. Sulphydryl groups are naturally found in the structure of only one amino acid: **cysteine**. Therefore, monomers, mAbs and Fab regions must contain free cysteines in their structures in order to attach them to liposomes via maleimide lipids. All of them possess cysteines as shown in figures 3.2 and 3.3. However not all the cysteines are free. This is the case for MHC/peptide molecules where 6 cysteines are present in each complex. Two are in the $\alpha 2$ domain at positions 101 and 164; two in the $\alpha 3$ domain at positions 203 and 259, and two in β_2m at positions 25 and 80, however none of them are free as they are used for inter-protein binding (Bjorkman, Saper et al. 1987). Therefore in order to achieve MHC class I binding and improve Ab and Fab binding, coupling was accomplished as follows: The proteins used (MHC class I monomers, Abs or Fab regions) were incubated with Traut's reagent: 2-Iminoethanol hydrochloride (Sigma-Aldrich) for 1hr at room temperature at a 20:1 Traut's:protein molar ratio in hydration buffer at pH 8. Traut's reagent reacts with primary amines ($-NH_2$) to introduce sulphhydryl ($-SH$) groups while maintaining charge and functional properties (PIERCE). This interaction creates "sticky" groups in amino acids which possess primary amines. All 20 amino acids possess primary amines, which will greatly enhance protein binding to maleimide lipids. Following activation of proteins with Traut's reagent, it was removed by passing the proteins through desalting columns as described in material and methods. Activated MHC class I monomers, mAbs or Fab regions were then incubated with the liposomes for

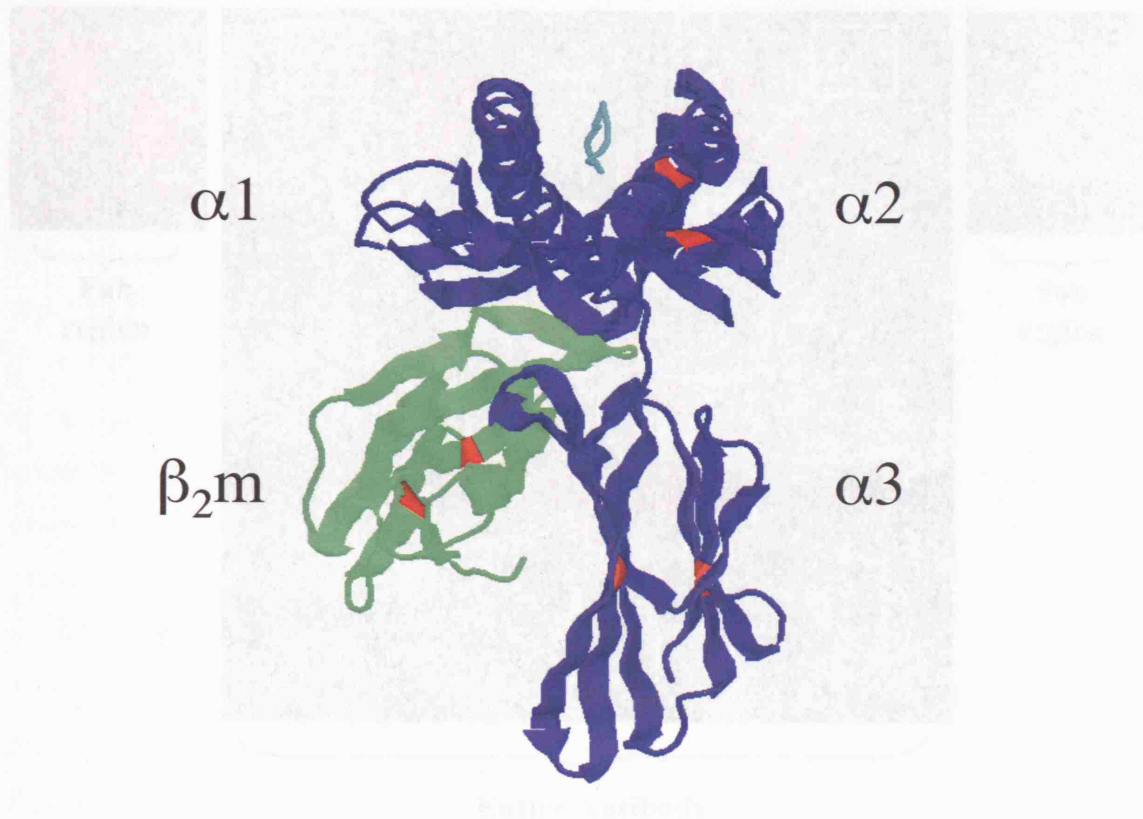


Figure 3.2.

CYSTEINE POSITIONS IN THE MONOMER STRUCTURE: There are six cysteines (in red) in the MHC class I monomer structure. Four in the A2 molecule (two in the $\alpha 2$ chain, positions 101 and 164 and two in the $\alpha 3$ chain, positions 203 and 259), and two in the β_2m at positions 25 and 80. These cysteines contain the sulphydryl groups, which are the binding site (when free) for the maleimide lipid incorporated on the surface of the liposomes.

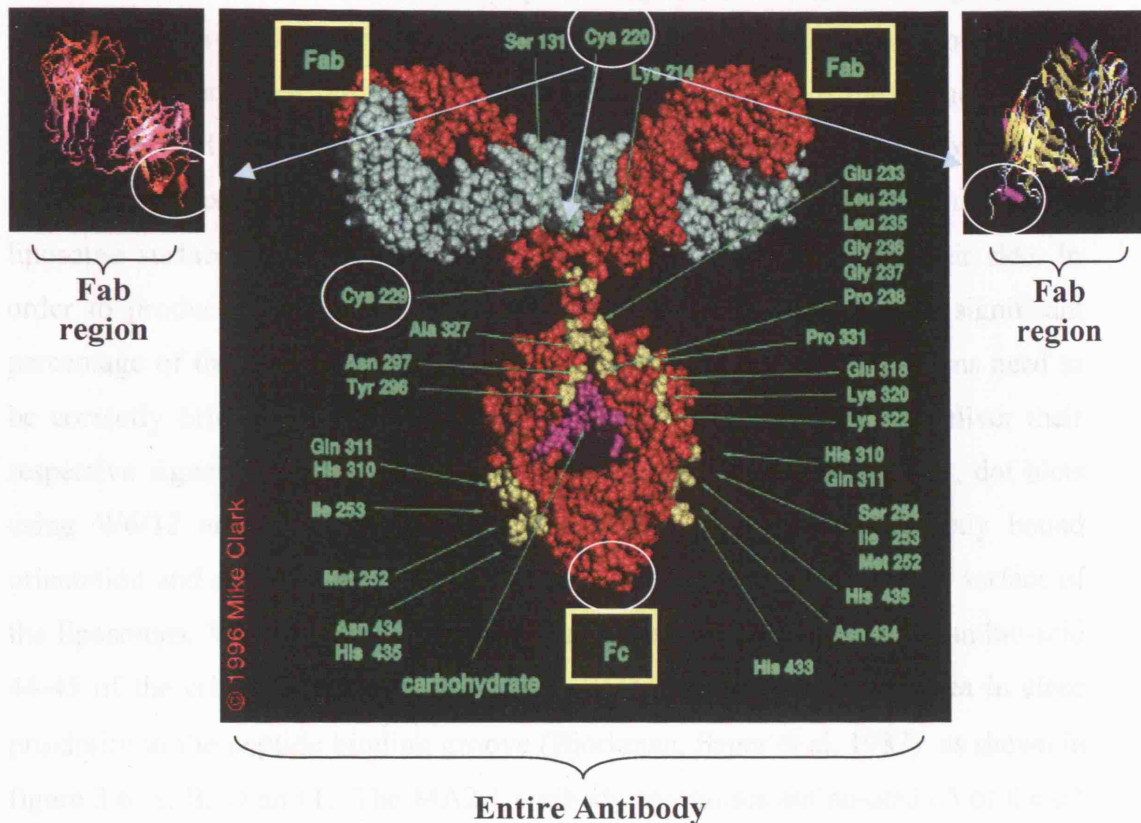


Figure 3.3.

CYSTEINE POSITIONS IN THE ANTIBODY AND FAB REGIONS STRUCTURE: Cysteines (white ovals) contain the sulphhydryl groups, which are the binding site for the maleimide lipid incorporated on the surface of the liposomes. Antibodies and Fab regions possess cysteines in their tails, allowing their antigen recognition sites to be free for interaction once bound to liposomes.

18 hrs at room temperature with constant mixing. Following coupling, coated liposomes were chromatographed through a CL-4B column (figures 3.4 and 3.5) to remove uncoupled monomers, Abs or Fabs regions and then concentrated, sterilised and stored at 4°C for further use as previously described. Even though protein binding is improved by activation with Traut's reagent, binding of proteins in a suitably orientated position could be compromised as many of the amino-acids in the structure of the proteins could be bound to maleimide lipids. Some MHC peptide complexes, antibodies and Fab regions could be effectively bound to the liposome surface while others could be bound upside-down or on their side. In order to produce a functional APC and deliver an efficient signal, a significant percentage of the MHC peptide complexes, antibodies and or Fab regions need to be correctly orientated on the surface of the liposomes in order to deliver their respective signals correctly. As explained subsequently in section 3.3.1, dot blots using W6/32 and MA2.1 antibodies were used to detect the correctly bound orientation and refolded structure of the MHC/peptide complexes on the surface of the liposomes. W6/32 antibodies recognise a monomorphic epitope on amino-acid 44-45 of the $\alpha 1$ domain of the MHC/peptide complex, which is an area in close proximity to the peptide binding groove (Bjorkman, Saper et al. 1987) as shown in figure 3.6 A, B, D and E. The MA2.1 antibody recognises amino-acid 65 of the $\alpha 1$ domain in closer proximity to the peptide binding groove (Parham P) as shown in figure 3.6 A, C, D and E. Positive results (section 3.3.1) would indicate that a percentage of the MHC peptide molecules were sufficiently well orientated following the rationale that if the peptide binding groove is accessible by W6/32 and MA2.1 antibodies, it will also be accessible for T cell receptor binding. In addition to this, figure 3.7 shows that coated liposome aAPCs bind to T cells in an antigen specific manner as explained in detail in section 4.2 and 4.2.4, which shows that even though some of the MHC peptides complexes could be in an incorrect orientation, a sufficient percentage of correctly orientated molecules were present allowing the coated liposome aAPCs to facilitate antigen specific T cell binding. Functional tests were then performed to ensure that correct signalling to antigen specific T cells was occurring as outlined in chapter 4.

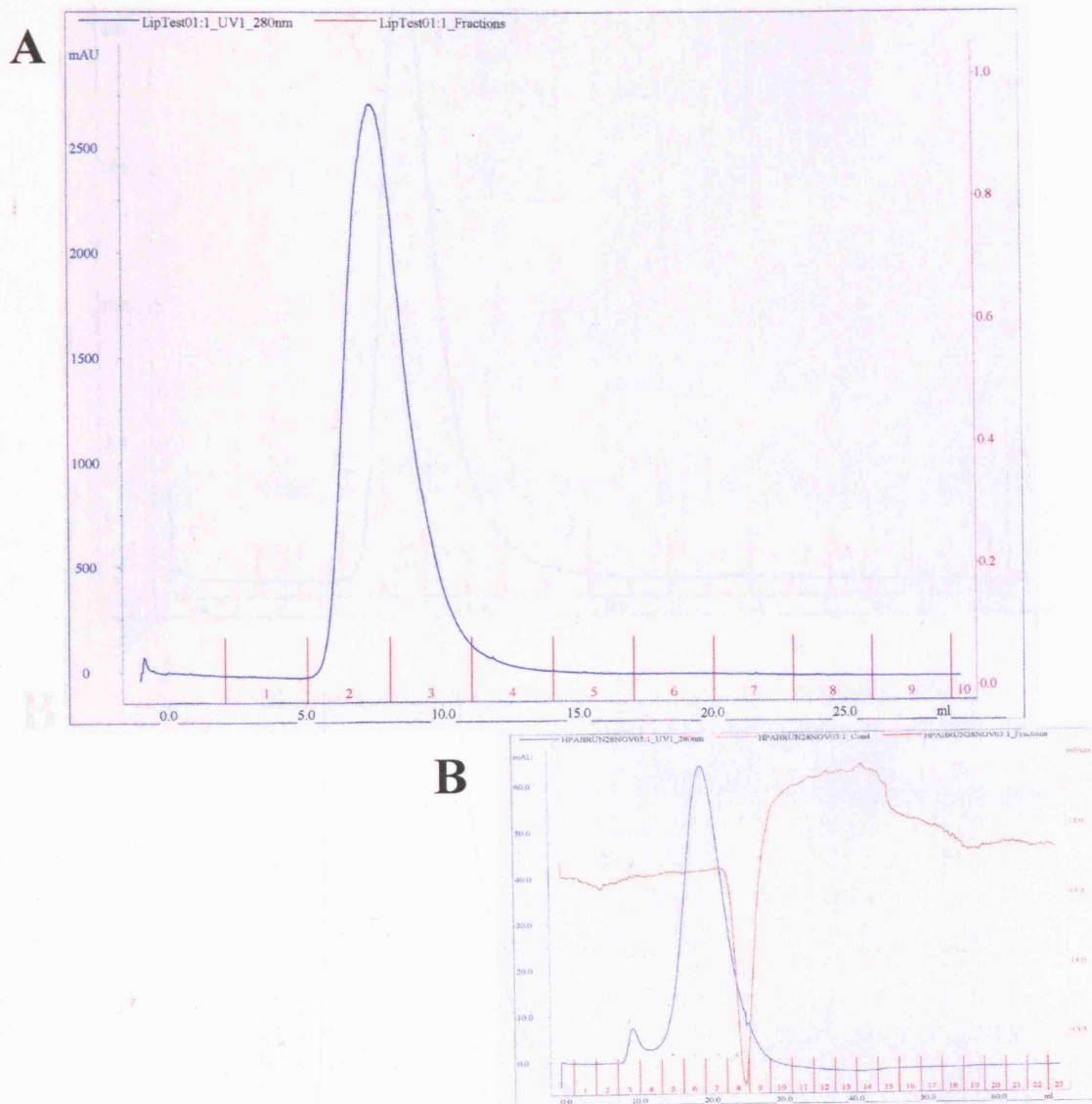


Figure 3.4.

LIPOSOME CL-4B CHROMATOGRAPHIC COLUMN PROFILE: **A.-** Liposomes run homogeneously in fractions two and three reflecting the quality and efficiency of the 100 nm extrusion. Liposomes are then recovered, concentrated and sterilised. **B.-** MHC class I monomers, antibodies and Fab regions themselves run (when free) in fractions 6, 7 and 8.

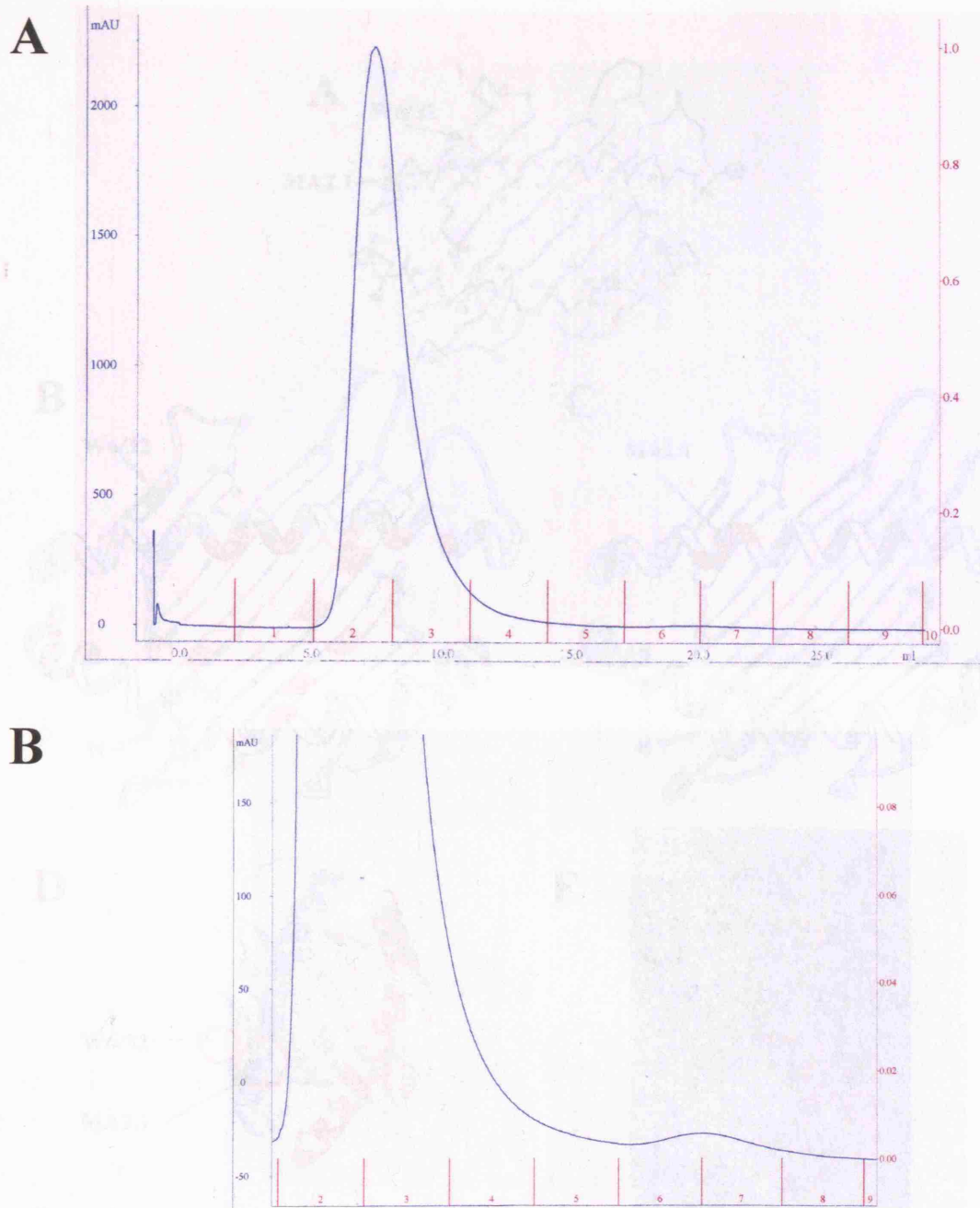


Figure 3.5.

LIPOSOMES, MHC MONOMERS, ANTIBODIES AND FAB REGIONS CL-4B CHROMATOGRAPHIC COLUMN PROFILE: **A.-** After 18 hrs of coupling, coated liposomes were passed through the CL-4B column to remove uncoupled monomers, Abs and/or Fab regions. Fractions 2 and 3 contain the coated liposomes whereas fractions 6 and 7 contain the uncoupled or free proteins (monomers, Abs and/or Fab regions). **B.-** Zoom of fractions 6 and 7 where a small peak of uncoupled protein can be recovered.

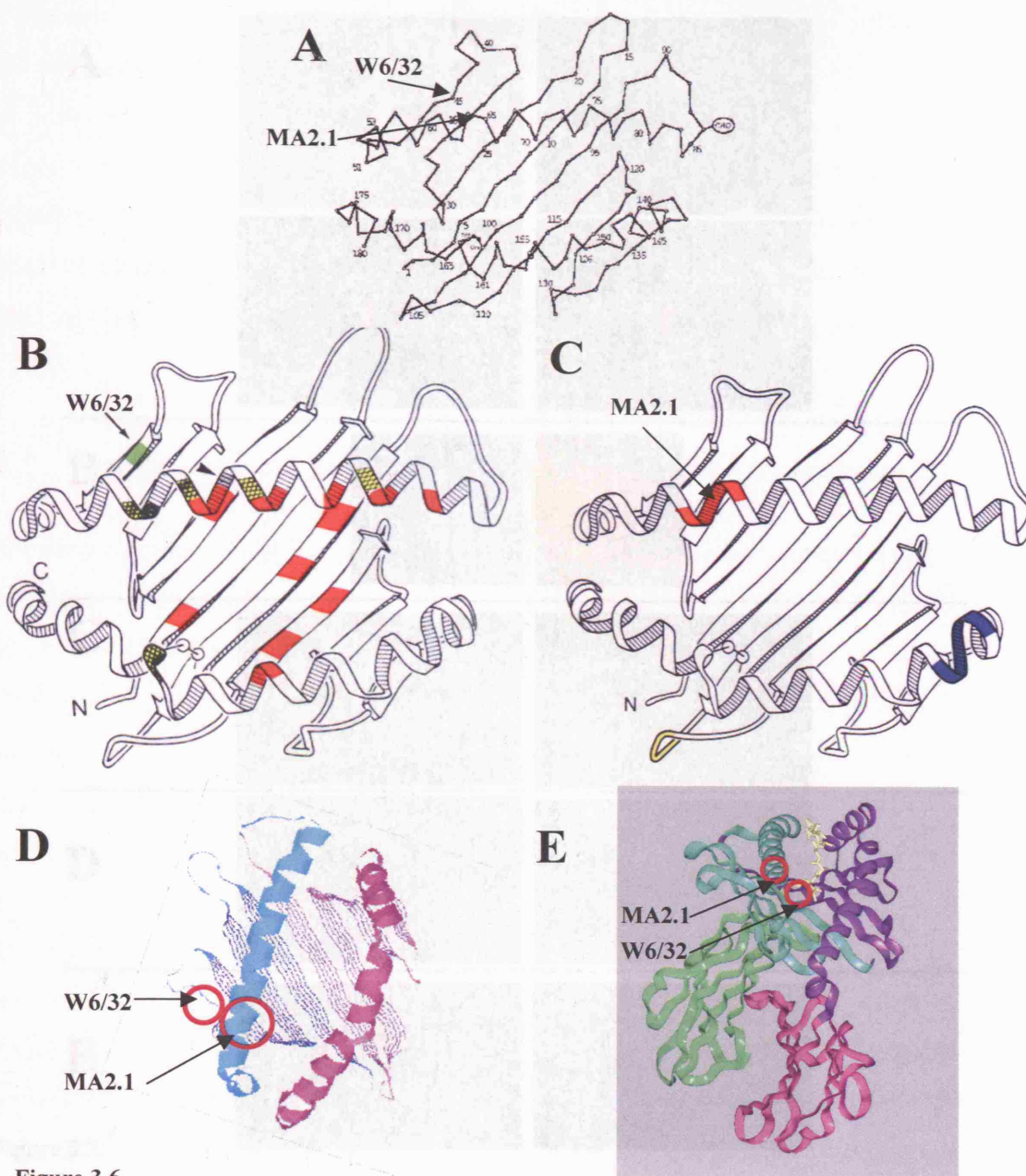
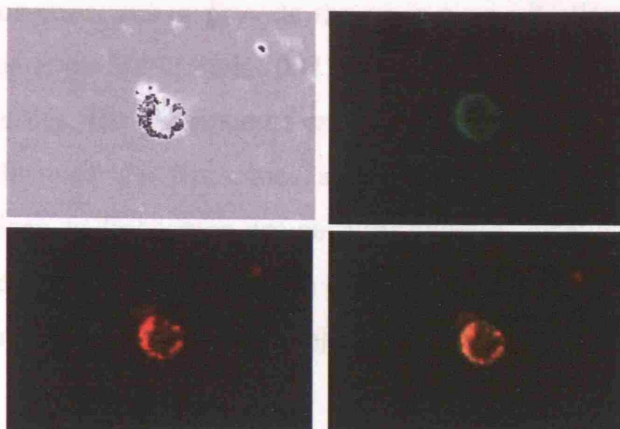


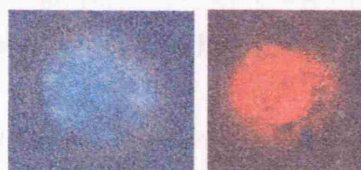
Figure 3.6.

ANTI CLASS I ANTIBODY RECOGNITION SITES: A, B, C and D.- Amino acid positions and antibody recognition sites for the A*0201 MHC class I molecule (seen from above.) W6/32 antibodies recognise positions 44 and 45 of the $\alpha 1$ chain whereas MA2.1 antibodies recognise position 65. Both sites in close proximity to the peptide binding groove. These antibodies were used to assess the orientation of the MHC peptide molecules on the surface of the liposomes. **E.** Coronal view of the MHC peptide complex.

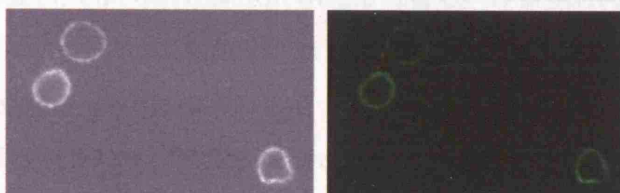
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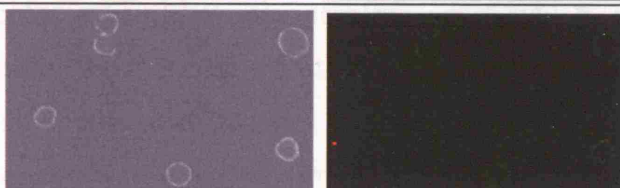
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D



E

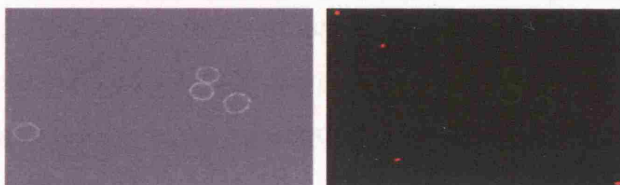


Figure 3.7.

LIPOSOME - T CELL ANTIGEN SPECIFIC BINDING: **A.-** Shows MHC peptide complex and co-activatory signal coated fluorescent liposomes in red bound to a CD8+ T cell (stained with FITC anti CD8). **B.-** Shows an APC-CMV tetramer positive T cell in blue covered with coated liposomes showing antigen specific binding. Since uncoated liposomes do not bind to T cells (**C**) and coated liposomes did not bind to T cells in a CMV matched/HLA mismatched culture (**D**) nor in an HLA matched/CMV mismatched culture (**E**), liposome - T cell binding was shown to be MHC/peptide dependent corroborating the correct orientation of the molecules on the liposome surface.

Antibodies and Fab regions have been previously shown to be correctly orientated on the surface of liposomes and to provide the correct transduction of signals by Park et al. (Park, Hong et al. 2002; Park 2002). In addition, as shown in figures 3.9 and 3.10 from our studies, the presence of antibodies against T cell co-activatory receptors on the surface of the liposomes at least doubled the level of T cell expansion in comparison to liposomes not carrying them as detailed in chapter 4, further confirming that the overall orientation of the molecules on the surface of the liposomes was predominantly correct ensuring the correct delivery of their signals to T cells.

3.3.1 Confirmation Of Liposome-MHC Class I Monomer Attachment

Following liposome-MHC monomer coupling, CL-4B column chromatographic purification, concentration and sterilisation of the aAPCs as described in materials and methods, dot blots were performed in order to assess effective monomer incorporation and presence on the surface of the liposomes (figure 3.8). These dot blots used W6/32 and MA2.1 antibodies separately. Both antibodies only recognise MHC class I molecules when they are correctly refolded. A positive result confirmed the presence of the monomers on the surface of the liposomes and their intact conformation after the construction process. Since W6/32 and MA2.1 antibodies recognise epitopes in close proximity to the peptide binding groove as previously mentioned, positive results indicate that a percentage of the MHC peptide molecules were well oriented on the surface of the liposomes. The system was also tested functionally as shown subsequently in chapter 4. However in order to check the stability of the MHC/peptide complexes and strength of liposome binding, coated liposomes were again passed through the CL-4B column 3, 5 and 7 days following the initial binding of MHC class I monomers to the liposomes in order to remove any detached monomers that had occurred in the intervening time. Dot blots were performed following these re-purification steps and the MHC peptide complexes were still detectable at all time points (fig 3.8) showing strong MHC-liposome binding. This is of particular importance as it shows that the aAPCs were sufficiently stable to maintain and deliver their signals for an adequate time

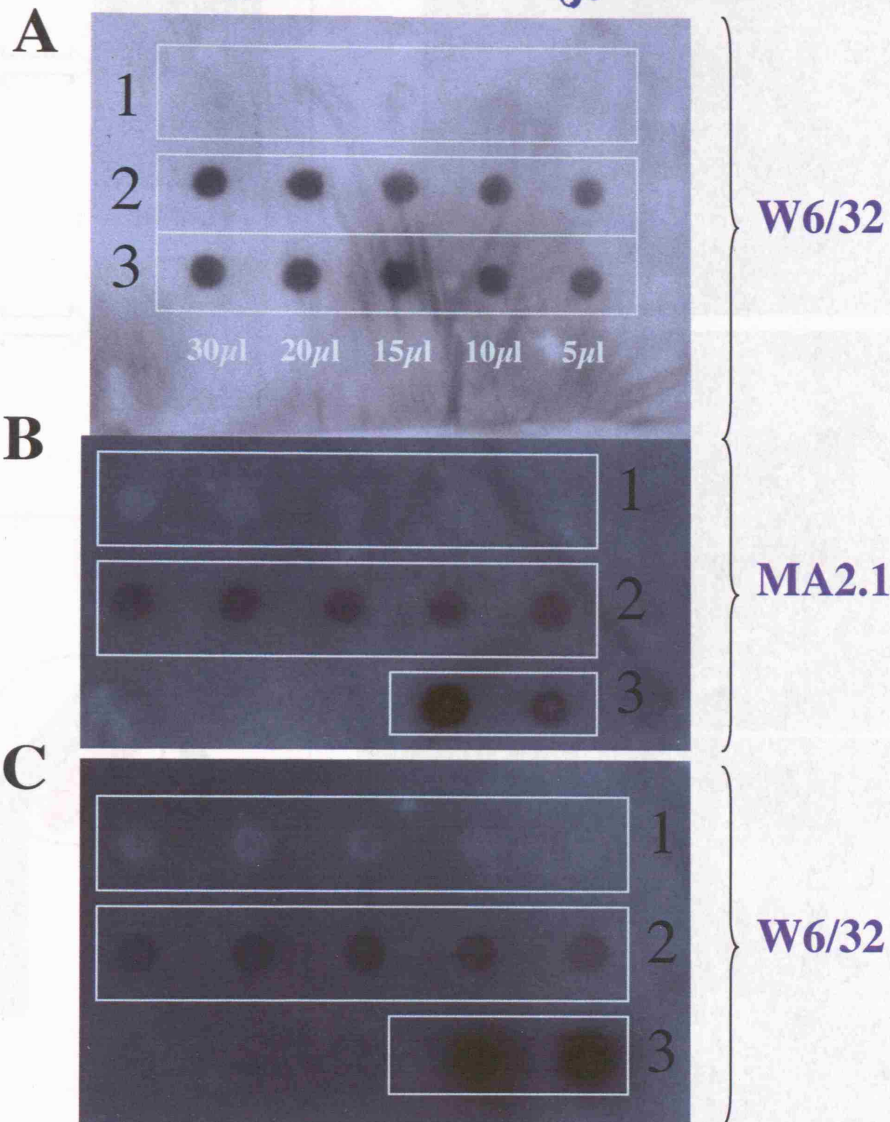
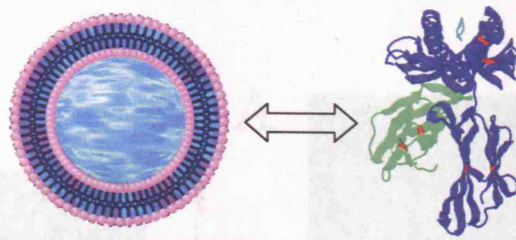


Figure 3.8.

CONFIRMATION OF MHC PEPTIDE COMPLEX-LIPOSOME ATTACHMENT BY DOT BLOT: **A.- W6/32 Dot Blot at day 2** following coupling: Line 1.- Increasing amounts of uncoated liposomes as negative controls. Lines 2 and 3.- Increasing amounts of MHC peptide complex coated liposomes after CL-4B column purification analysed in duplicates to detect the presence of well refolded MHC class I monomers on the surface of the liposomes. **B.- MA2.1 Dot Blot at day 2** following coupling. Line 1.- uncoated liposomes as negative controls. Line 2.- MHC peptide complex coated liposomes after CL-4B column purification and line 3.- MHC peptide complexes themselves as positive controls. **C.- W6/32 Dot blots at day 7** following coupling. Line 1.- uncoated liposomes as negative controls. Line 2.- MHC peptide complex coated liposomes after CL-4B column purification to separate detached monomers over time, and line 3.- MHC peptide complexes as positive controls. Monomers remained correctly refolded and attached on the surface of

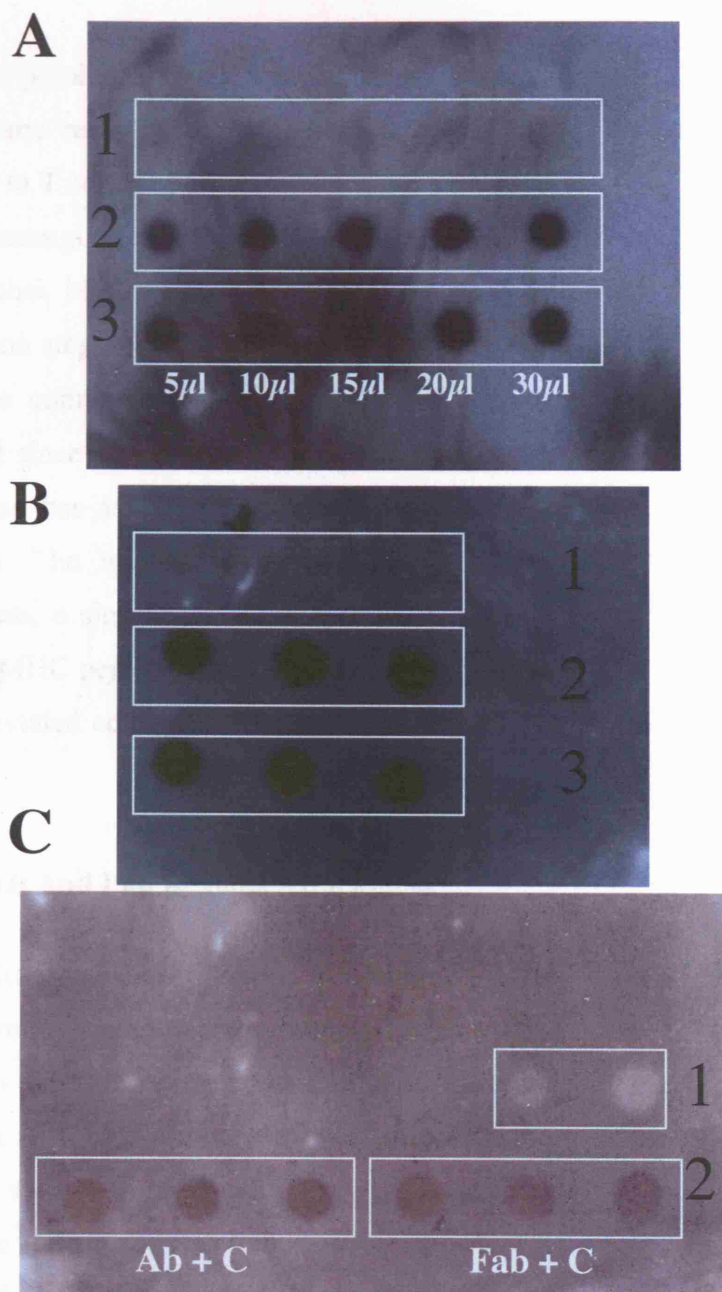
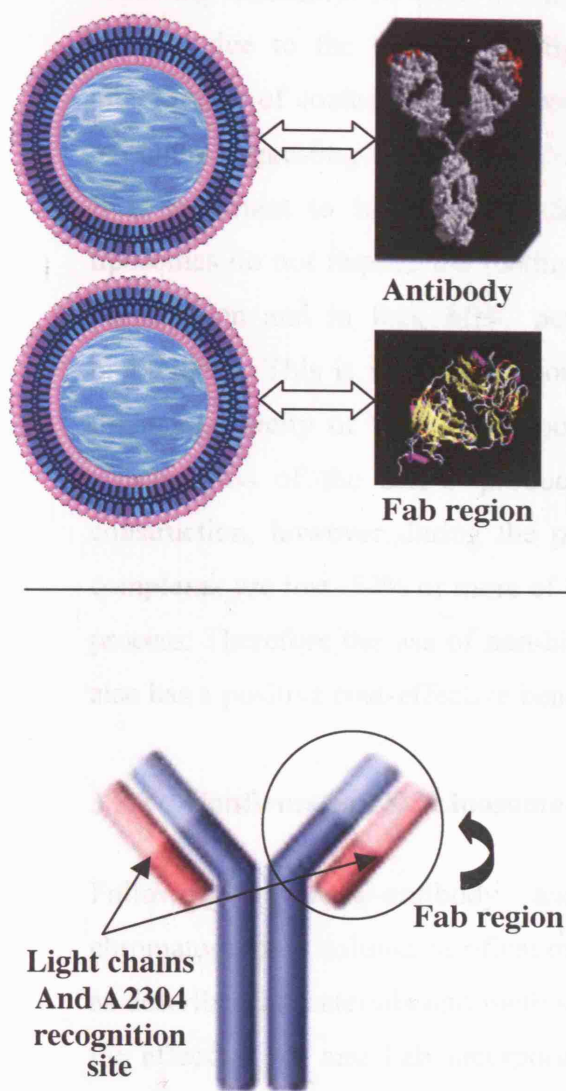


Figure 3.9.

CONFIRMATION OF AB AND FAB - LIPOSOME ATTACHMENT BY DOT BLOT: **A.-** Anti Fab A2304 antibody dot blot detection at day 2 following coupling. Line 1.- Increasing amounts of uncoated liposomes as negative controls. Line 2.- Increasing amounts of antibody coated liposomes after CL-4B column purification and 3.- Increasing amounts of Fab coated liposomes after CL-4B column purification analysed to detect the presence of Fab regions on the surface of the liposomes. **B.-** Anti Fab dot blots at day 14 following coupling. Line 1, uncoated liposomes as negative controls. Line 2.- antibody coated liposomes and line 3.- Fab region coated liposomes after CL-4B column purification to separate detached antibodies or Fab regions over time. **C.-** Controls: Line 1.- uncoated liposomes as negative controls and line 2.- left hand side dots: Abs as positive controls and right hand side dots: Fab regions as positive controls. Antibodies and Fab regions remained bound to the surface of the liposomes for up to 14 days.

following assembly. *In vivo*, it was not possible to fully test the liposome-MHC stability due to the problem of liposome recovery once injected, however the micrographs of coated liposomes bound to T cells in fig 3.7 were taken after 4 days of culture suggesting that the aAPCs possess good stability in cell cultures.

It is important to highlight the fact that MHC/peptide complexes attached to liposomes do not require the biotinylation step discussed in chapter 2 for tetramer construction and in fact, MHC peptide complexes bound to liposomes are not biotinylated. This is not only important since biotin could affect the non-specific immunogenicity of the aAPCs, but also has an impact on the timing and cost-effectiveness of the aAPC production. The biotinylation step allows tetramer construction, however during the process, a significant amount of MHC peptide complexes are lost. 50% or more of the MHC peptide complexes are lost during the process. Therefore the use of non-biotinylated constructs for the APC construction also has a positive cost-effective benefit.

3.3.2 Confirmation Of Liposome-mAb And Fab Regions Attachment

Following liposome-antibody and liposome-Fab region coupling, CL-4B chromatographic column purification, concentration and sterilisation of the aAPCs as described in materials and methods, a dot blot was performed in order to assess the effective Ab and Fab incorporation and their presence on the surface of the liposomes (figure 3.9). This dot blot was performed with the A2304-SIGMA antibody that exclusively recognises the light chains of Fab regions. Therefore a positive result confirmed the presence of Abs and Fab regions on the surface of the liposomes and provided an indication of the orientation of the molecules. There is however extensive data from *in vivo* studies, addressing the correct orientation and proper signalling of antibodies and Fab regions on coated liposomes (Park, Hong et al. 2002; Park 2002) as mentioned previously. Figure 3.10 from our results showed that antibodies against T cell co-activatory receptors on the surface of the liposomes had a significant impact on T cell expansion, implying that the overall orientation of the molecules on the surface of the liposomes was correct and that they were able to deliver signals correctly. However in order to check the stability and strength of the

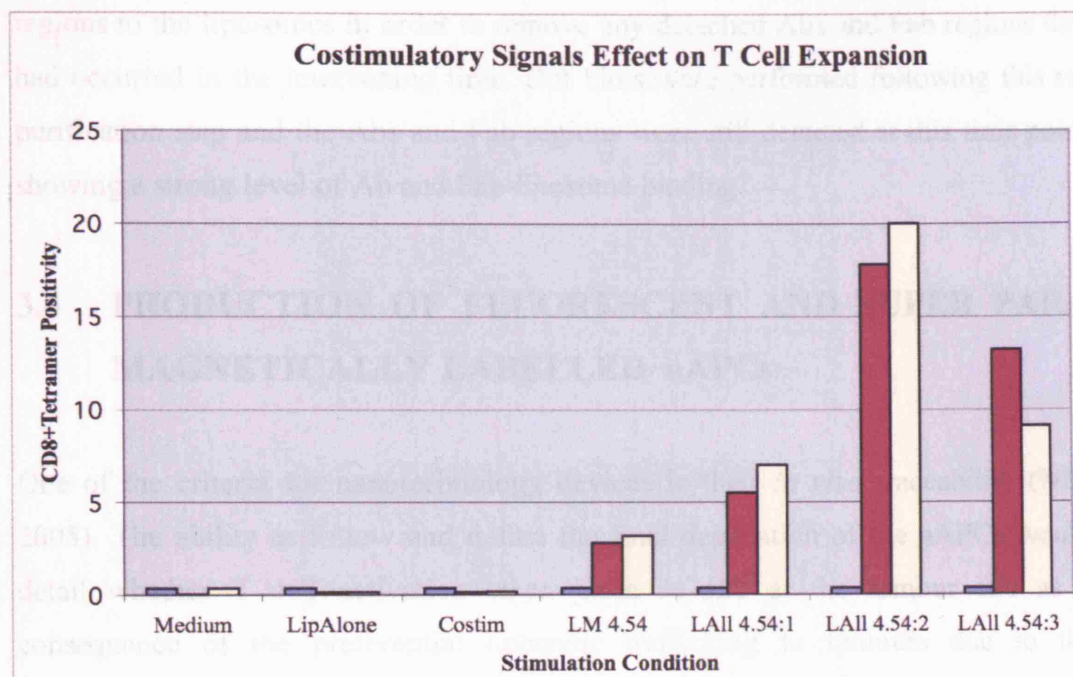


Figure 3.10.

CO-ACTIVATORY SIGNALS EFFECT ON T CELL EXPANSION: PBMCs were stimulated with medium, uncoated liposomes (Lip Alone), liposomes coated with all the adhesion and co-activatory signals (anti LFA1, anti CD28, anti CD27, anti CD40L and anti 4-1BB), but without MHC peptide complexes (Costim), liposomes coated exclusively with 4.54 μ g of MHC peptide complexes and coated liposomes with 4.54 μ g of MHC peptide complexes plus 1 μ g, 2 μ g and 3 μ g of each of the co-activatory signals respectively. The addition of 1 μ g of each of the co-activatory signals nearly doubled the level of T cell expansion when compared with the expansion obtained by PBMCs stimulated with liposomes coated exclusively with MHC peptide complexes. 2 μ g of each of the co-activatory signals dramatically increased the level of T cell expansion. This figure shows that co-activatory signals as antibodies or Fab regions on the surface of the liposomes were well orientated and signalling correctly following binding. 2 μ g of each of the co-activatory signals delivered the best expansion, therefore this was chosen as optimal dose in future experiments. It is believed that 3 μ g of each of the co-activatory signals induced T cell over stimulation and death.

Ab – liposome and Fab – liposome binding, coated liposomes were again passed through the CL-4B column 14 days following the initial binding of Ab and Fab regions to the liposomes in order to remove any detached Abs and Fab regions that had occurred in the intervening time. Dot blots were performed following this re-purification step and the Abs and Fab regions were still detected at this time point showing a strong level of Ab and Fab-liposome binding.

3.4 PRODUCTION OF FLUORESCENT AND SUPER PARA MAGNETICALLY LABELLED aAPCs

One of the criteria for nanotechnology devices is their *in vivo* traceability (NIH 2005). The ability to follow and define the final destination of the aAPCs would detail whether T cell activation takes place *in situ* at the tumour site as a consequence of the preferential liposome trafficking to tumours due to the previously explained EPR effect. Traceable aAPCs would help to demonstrate whether activation of naïve T cells takes place in lymph nodes. *In vivo* follow up will provide information about their presence, organ trafficking and half-life of the system. Having a traceable product would be useful in helping to identify cell-aAPC interactions in *in vitro* cell cultures. Therefore in order to produce traceable aAPCs, liposomes were produced double labelled.

3.4.1 Fluorescent Liposomes

Initially the rhodamine labelled fluorescent lipid was added to the mixture in order to make fluorescent liposomes (figure 3.11). In doing so, we were able to follow the liposomes in *in vitro* cultures and in tissues following post-mortem biopsies. Details of the cell-aAPC *in vitro* interactions will be described in chapter 4. The liposomes however also needed to be traceable in real time *in vivo* as the system was intended to be used for active immunotherapy in order to mount immune responses *in vivo*.

3.4.2 Super Paramagnetic Liposomes (Magnetoliposomes)

In following nanotechnology applications are closely monitored as they develop devices to enhance existing "spion" systems. By characterizing properties synthesized following University of Nottingham

A



B

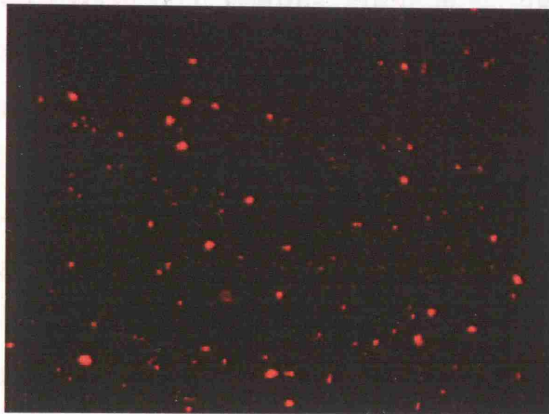
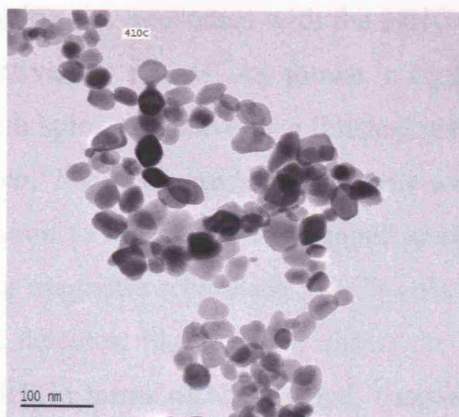
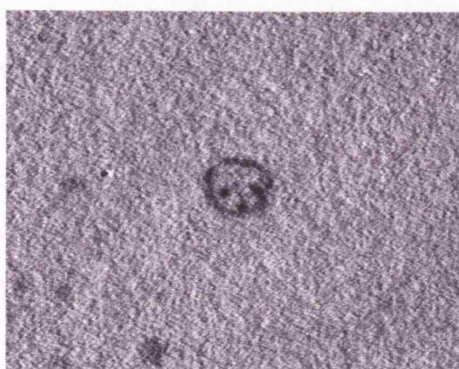
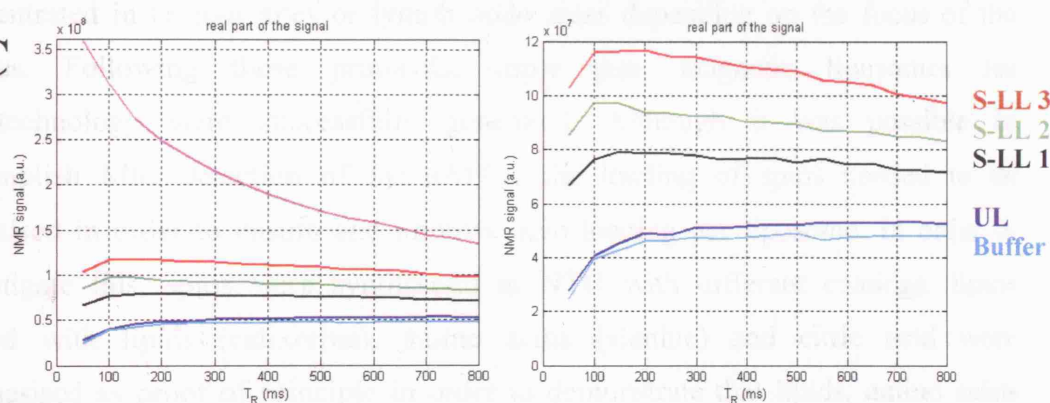


Figure 3.11.

FLUORESCENT LIPOSOMES: In order to generate a traceable aAPC, DPE-rhodamine was incorporated in the liposomes to allow their visibility in fixed *ex-vivo* and *in-vivo* samples. **A.-** Fluorescent liposome batches (normal scale) and **B.-** Fluorescent liposomes under fluorescence microscopy.

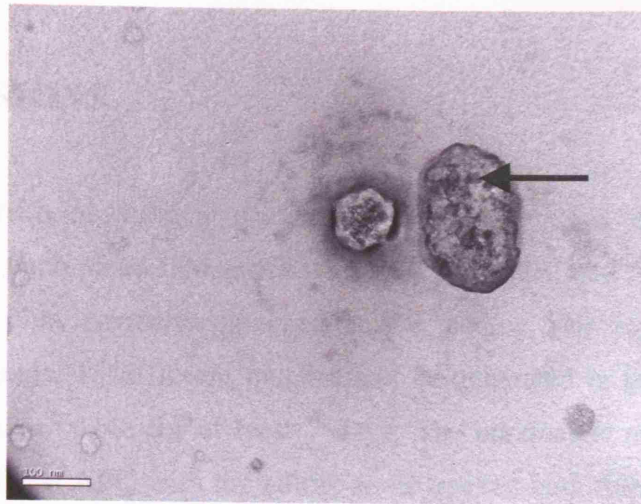
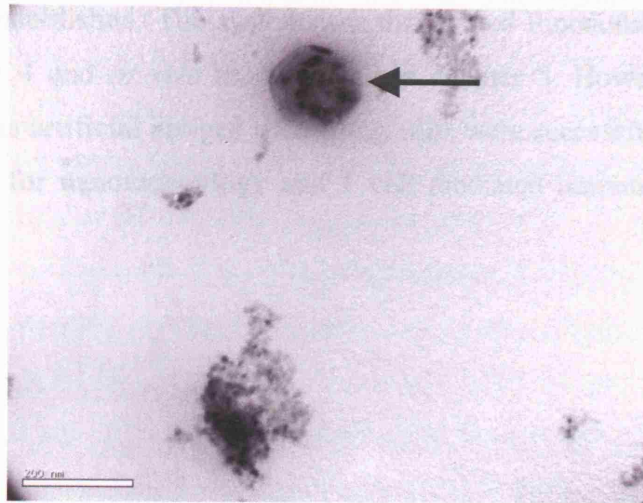
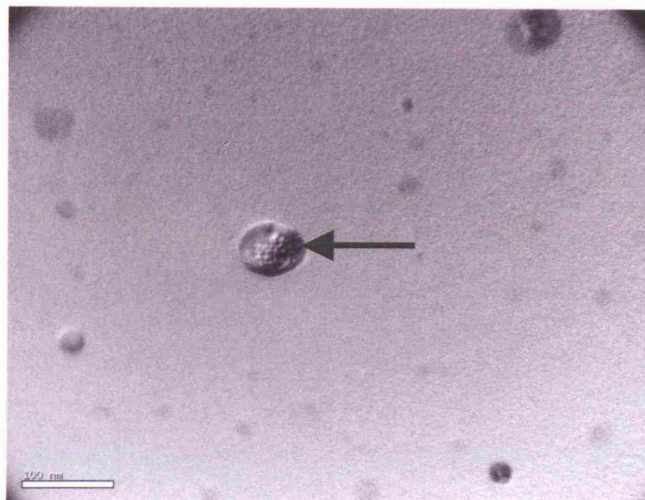
3.4.2 Super Para Magnetic Liposomes (Magnetoliposomes)

In following nano devices, expected and unexpected interactions are closely monitored as they develop. Therefore and even though different strategies to trace devices *in vivo* exist, the use of super para magnetic iron oxide nano particles or “spios” became the obvious choice to accomplish this due to their size and characteristic properties. Spios have a 5 to 13 nm size (Figure 3.12A). They were synthesised following a standard protocol (Khalafalla 1980) at Nottingham Trent University (NTU) by Dr Gareth Cave (Chemistry Department). Spios are of particular importance since they can be detected by magnetic resonance imaging (MRI) (Bulte and De Cuyper 2003; Martina, Fortin et al. 2005) and also because they have been shown to be capable of being concentrated in particular body areas using external magnetic fields as they are super para magnetic (Babincova, Altanerova et al. 2000; Fortin-Ripoche, Martina et al. 2006). Super para magnetic particles are not magnetic themselves. However they become magnetic when magnetic external fields are applied to them. Spios were chosen for incorporation since they are firstly safe, which is a critical requirement for an *in vivo* strategy. Spios were first described in bacteria (Bazylinski and Frankel 2004). It has been suggested and proven in some cases that all species produce spios as a way of effecting spatial magnetic orientation especially in migrating birds and fish. Spios have also been found in the human brain (Kirschvink, Kobayashi-Kirschvink et al. 1992) and even though their exact function in humans has not been determined, it is known that macrophages transform them and excrete them as part of the iron content of the “haem” groups of haemoglobin. Once it was found that they were safe, spios have been used *in vivo* in humans as a means to enhance magnetic resonance imaging studies (Bulte, de Cuyper et al. 1999) and as part of cancer immunotherapy strategies (Ito, Kuga et al. 2004; Hu, Kettunen et al. 2005). As mentioned previously, liposomes have liquid interiors. Therefore, it was decided to insert spios into the liposomes, which would make the liposomes traceable *in vivo* using MRI scans and their surfaces would still be intact for carrying the signals for T cell engagement. Therefore liposomes were either sonicated or hydrated in the

A**B****C****Figure 3.12.**

MRI *IN-VIVO* TRACABLE aAPC: Super para magnetic iron oxide nano particles or spios (**A**) can be detected by magnetic resonance imaging (MRI) and can be concentrated to specific areas by using magnetic fields. In order to create an *in-vivo* traceable aAPC, liposomes where loaded with spios. **B.-** Shows a liposome containing 3 spio particles viewed by electron microscopy. Following chromatographic separation of non entrapped spios, loaded liposomes were concentrated using magnetic columns and analysed on a mini MRI detector (**C**). The right hand side graphic is a zoom of the one on left hand side. Buffer and unloaded liposomes (UL) did not emit any MRI signal whereas spios-loaded liposomes (S-LL) in three increasing concentrations emitted a strong and powerful signal allowing the artificial APCs to be detected by MRI.

presence of spios before coating the liposomes with the MHC / peptide complexes and antibodies against co-activatory signals. As shown in figure 3.12B, liposomes were successfully loaded with spios, this gave us a finished product that was dually labelled and traceable *in vivo*. The liposome/spio mixtures were passed through a CL-4B chromatography column to remove non-entrapped spios. Loaded liposomes were then concentrated using magnetic separation MACS columns and analysed by MRI using first a mini MRI detector. Figure 3.12C shows the MRI signal detected from loaded liposomes, which in terms of MRI signal, translates into a very large and powerful signal emission. *In vivo* studies were then performed to establish the performance of our constructs. MRI *in vivo* evaluation and detection as well as *in vivo* kinetics were also studied and will be described in detail in chapter 5. As loaded liposomes are super para magnetic, this not only allows their *in vivo* detection using this non-invasive MRI technology, but also allows their magnetic targeting to particular body areas when external magnetic fields are applied as described previously. Therefore the artificial APCs could eventually be concentrated in tumour sites or lymph node areas depending on the focus of the studies. Following these protocols, super para magnetic liposomes for nanotechnology were successfully generated. Although it was possible to accomplish MRI detection of the aAPCs, the loading of spios needed to be optimised in order to ensure and improve spio loading per liposome. In order to investigate this, spios were synthesised at NTU with different coatings. Spios coated with lipids (calixerine), amino acids (alanine) and citric acid were synthesised as proof of principle in order to demonstrate that lipids, amino acids and citric acid, which prevent spios clustering, could be used with the spios and facilitate the loading of liposomes with greater numbers of spios. Figure 3.13 shows that liposomes were loaded with larger amounts of spios when using coated nanoparticles.

A**B****C****Figure 3.13.**

SPIOS-LIPOSOME LOADING IMPROVEMENT: Super para magnetic iron oxide nano particles or spios were coated with calixerine (**A**), alanine (**B**) or made in citric acid (**C**) as a means to further improve spios-liposome loading, as normally around 10% of liposomes entrap spios. A, B and C show electron microscope pictures of liposomes containing increased number of spios after addition of these lipophilic components doubling at least the loading.

3.5 CONCLUSIONS

In summary, an artificial antigen presenting cell system was developed by successfully coating nano-sized liposomes with well orientated and refolded MHC peptide complexes plus correctly attached mAbs and/or Fab regions as co-activatory T cell signals. 15 different batches can be generated in parallel in less than 48 hrs and remain stable for at least 7 days. The parameters needed for the generation of an artificial APC with the ability to be tracked both fluorescently and by MRI have been established. The system was then tested functionally *ex vivo* as described in chapter 4 and *in vivo* as described in chapter 5. However immuno-magneto-liposomes as artificial antigen presenting cells were successfully generated and a new platform for nanotechnology and T cell mediated immunotherapy was constructed.

CHAPTER 4

aAPC FOR ADOPTIVE IMMUNOTHERAPY

4.1 INTRODUCTION

The infusion of T cells to patients known as T cell adoptive immunotherapy, is now a common practice clinically (Oelke, Krueger et al. 2005) as a result of encouraging results. For some human tumours such as chronic myeloid leukaemia (CML) for instance, T cell adoptive immunotherapy in the form of Donor Lymphocyte Infusions (DLI) has been proven not only to rescue patients after relapse (Drobyski, Keever et al. 1993), but also able to effect a complete cure (Kolb, Mittermuller et al. 1990; Mackinnon, Papadopoulos et al. 1995; Falkenburg, Wafelman et al. 1999). T cell adoptive immunotherapy has also been shown to be very effective against some viral infections such as CMV (Peggs and Mackinnon 2002; Peggs, Verfuerth et al. 2003) in patients who are particularly vulnerable after bone marrow transplantation (BMT) due to their immunosuppressive and chemotherapeutic regimes that these patients are given. T cell adoptive immunotherapy is evolving from the administration of unsorted T cell populations to the administration of sorted antigen specific T cell populations (Cobbold, Khan et al. 2005) as will be explained in detail. It has to be borne in mind however, that for CML, it is still not clear exactly which subset(s) of cells from the unsorted T cell population are responsible for the eradication of the disease. CD8 positive T cells are one of the “primary suspects” of the graft versus leukaemia effect (GvL) observed by DLIs in CML patients since purified CD8+ DLIs are capable of inducing remission. Unfortunately graft versus host disease (GvHD) is a collateral event and sometimes the GvHD effect develops before any GvL effect can be detected. Having said that, CD4+ DLIs or CD8 depleted DLIs have also been shown to induce remission in CML patients, whether the GvL effect is mediated directly by CD4 T cells by killing of targets or whether CD4 T cells provide some critical “missing help” to the CD8 T cells already present

in the patients remains to be determined. These results however demonstrate that T cells play a protagonist role in anti tumour responses. In the case of CMV or in other instances where the target antigen(s) is (are) well known, sorted antigen specific T cells are selectively administered instead. In doing so, the anti viral effect remains whereas GvHD is reduced to a minimum (but not completely removed). However even when effective anti tumour and anti viral responses in the absence or presence of tolerated GvHD are generated by the infused cells, adoptive immunotherapy faces a problem; the *in vivo* survival of T cells is sometimes compromised by the *ex vivo* culture required to expand the cells in order to produce and deliver significant impact in the patients, and it seems that the longer the *in vitro* T cell culture the shorter the *in vivo* T cell survival after infusion (Dudley and Rosenberg 2003). Therefore more recently, alternative infusion strategies have been explored. Using tetramers and magnetic beads, Cobbold et al, isolated anti CMV specific CD8 T cells from donors followed by immediate infusion into patients without any *ex vivo* expansion. This strategy has been used in bone marrow transplant recipients who have experienced CMV reactivation (Cobbold, Khan et al. 2005). In doing so, this strategy "sacrifices" a high final T cell number in exchange for T cell *in vivo* survival after infusion by reducing as much as possible the *in vitro* culture period. The protective effect observed by this theoretically "small" number of antigen specific T cells following infusion, occurs after Bone Marrow Transplantation (BMT) partially because there is a "privilege empty environment" in the patient following extensive chemotherapy and total body irradiation, which allows infused T cells to replicate much more rapidly. It is not possible or very difficult to reproduce this phenomenon in other cancer patients since they do not share the empty environment produced by systemic chemotherapy and radiotherapy. Even though this short culture / T cell sorting strategy is feasible and promising for anti viral treatment after transplant, with solid tumours, unfortunately donors are not available from which to purify tumour reactive T cells. It has been hypothesised however, that donors could be vaccinated with tumour peptides in order to mount anti-tumour responses in healthy individuals, which could then be utilised to source T cells for patients. This approach however will probably never

reach the clinical stage since healthy individuals could be put at risk as the immune response generated in a healthy individual against the tumour peptide could become tolerogenic rather than immunogenic, subsequently if the individual developed a tumour, it might be seen and recognised as self rather than foreign by his own immune system, as a consequence the tumour would be protected from immune killing. Currently however, adoptive immunotherapy is evolving towards the infusion of selected subsets of T cells in order to separate as much as possible anti tumour or anti viral responses from anti host responses. Another example of this is the selection of antigen specific / IFN- γ positive T cells (Becker, Pohla et al. 2001; Pittet, Zippelius et al. 2001). In isolating anti viral or anti tumour CD8+ IFN- γ + T cells for adoptive therapy, terminal effector and effector memory T cells are selectively administered. These cells should theoretically kill their relevant target and eventually die soon after since they are committed cytotoxic effectors according to their phenotype. Even though these cells have been found to be capable of reverting their phenotype towards less terminal functions (Wills, Okecha et al. 2002), there are concerns about not producing any immunological memory with this approach, this may or may not be a drawback depending on whether the tumour or viral infection carries the risk of relapse. It does however demonstrate that T cell adoptive therapy is evolving and moving forward by developing different infusion strategies. Nevertheless in order to avoid the use of donors as a source of cells and in order to avoid the *ex vivo* manipulation of T cells that can compromise the functional properties of the cells following infusion, *in vivo* artificial APCs with the potential of mounting immune responses within the patients themselves would be ideal in order to accomplish what is known as active immunotherapy. This approach is feasible if the memory or naïve T cells in the patients have not been deleted or anergised as a consequence of immune evasion and suppression mechanisms, which may be responsible for the development of the disease itself in the first place. Active immunotherapy strategies and the use of artificial APCs for active immunotherapy will be discussed in chapter 5. Adoptive immunotherapy however, remains in some instances as the only alternative for those patients in which T cells have been deleted, killed or anergised. Therefore adoptive immunotherapy will

hopefully be part of the future use of artificial APC systems since the expansion of T cells in a more reproducible, superior and comprehensive manner still remains as one of the issues for significant improvement despite the fact that adoptive immunotherapy is currently a therapeutic tool that already delivers significant clinical impact in patients. This is one of the main reasons for the creation of a novel artificial nano APC system. In order to address the potential of the system, once the nano artificial APCs were generated and having defined that they were structurally correct (chapter 3), the first objectives as to define whether the constructs were capable of delivering stimulatory signals following T cell co-cultivation and whether the system would be able to expand functional T cells *ex vivo* in a reliable, efficient and improved manner. The first critical step was to define the optimal dose of coated liposome APCs required to trigger T cell expansion as well as the quantity of each signal needed to deliver an appropriate stimulatory effect.

4.2 T CELL EXPANSION IN A MEMORY SETTING (CMV POSITIVE INDIVIDUALS)

The primary signal delivered by the MHC/peptide complex is the requirement for antigen specific T cell activation. For naïve T cells, MHC peptide recognition in the absence of co-activatory signals normally leads to clonal deletion, anergy or tolerance as previously described (Jenkins 1994; Johnson and Jenkins 1994). Activated and memory T cells however, respond, expand and kill following MHC peptide recognition even in the absence of co-activatory signals (Kohrt, Shu et al. 2005), their effect however, is magnified in the presence of co-activatory signals (Bukczynski, Wen et al. 2005). Therefore initially, 2 million PBMCs from an A2⁺B7⁻ (CMV positive) individual were stimulated with liposomes coated with increasing amounts of the relevant A2/CMV pp65 peptide complex (monomers). The levels of monomers coating the liposomes were selectively chosen according to the calculations discussed in chapter 2 without exceeding the maximum amount of protein that the liposomes could theoretically bind. Figure 4.1 shows that there is a

dose-dependent correlation linked to an increased T cell expansion according to the amount of monomers delivered on the liposomes in the absence of co-activatory signals. At this point, a high level of expansion was not the main objective since MHC peptide complexes were delivered in the absence of co-activatory signals. However the increasing levels of expansion obtained suggested that the artificial APCs were delivering the MHC peptide complexes appropriately and were generating T cell specific expansion. The proposed mechanisms of action by coated liposomes on T cells will be discussed in section 4.2.4, however we then looked at the effect on the addition of co-activatory signals on the liposomes in order to enhance the artificial system and the T cell stimulation. Following stimulation of PBMCs and CD8 purified T cell populations plus the irradiated CD8 T cell-depleted fraction, antigen specific expansions of 55 to 200 fold were reached when liposomes coated with both MHC peptide complexes and co-activatory signals were used (figure 4.2). The use of NLV-CMV relevant and irrelevant (SLK-CML-A2-restricted peptide) tetramers confirmed the specificity of the expanded cells. These levels of expansion were much higher than those achieved with other systems that we have previously used at the Anthony Nolan Research Institute for the same purpose such as peptide-pulsed dendritic cells (30 fold) figure 4.3, which is one of the standard methods currently used, commercial beads (not antigen specific) figure 4.4, and Daudi cells (40 fold) (Barber, Jordan et al. 2006). As shown in figure 4.2, either one or two μg of each one of the adhesion (anti LFA-1) and co-activatory molecules (anti CD28, anti CD27, anti CD40L and anti 4-1BB) in the form of monoclonal antibodies were incorporated on the surface of the liposomes in combination with MHC peptide complexes using some of the best and highest conditions (4.54 μg , 5.32 μg and 7.98 μg of MHC/peptide complexes) from the previous experiment in figure 4.1 using the optimal dose (1200 μl). One and two μg of mAbs translate to a 1.8:1.5 and 1.8:3 MHC/mAb molar ratio respectively for each co-activatory signal or to a 17 and 8.5 MHC/peptide molecules per each co-activatory signal respectively. On the 10 day plot with the pink asterisk in figure 4.2A (17.8%), two separate NLV specific population can be seen, a binding phenomenon that was found many times in different cultures suggesting that aAPCs

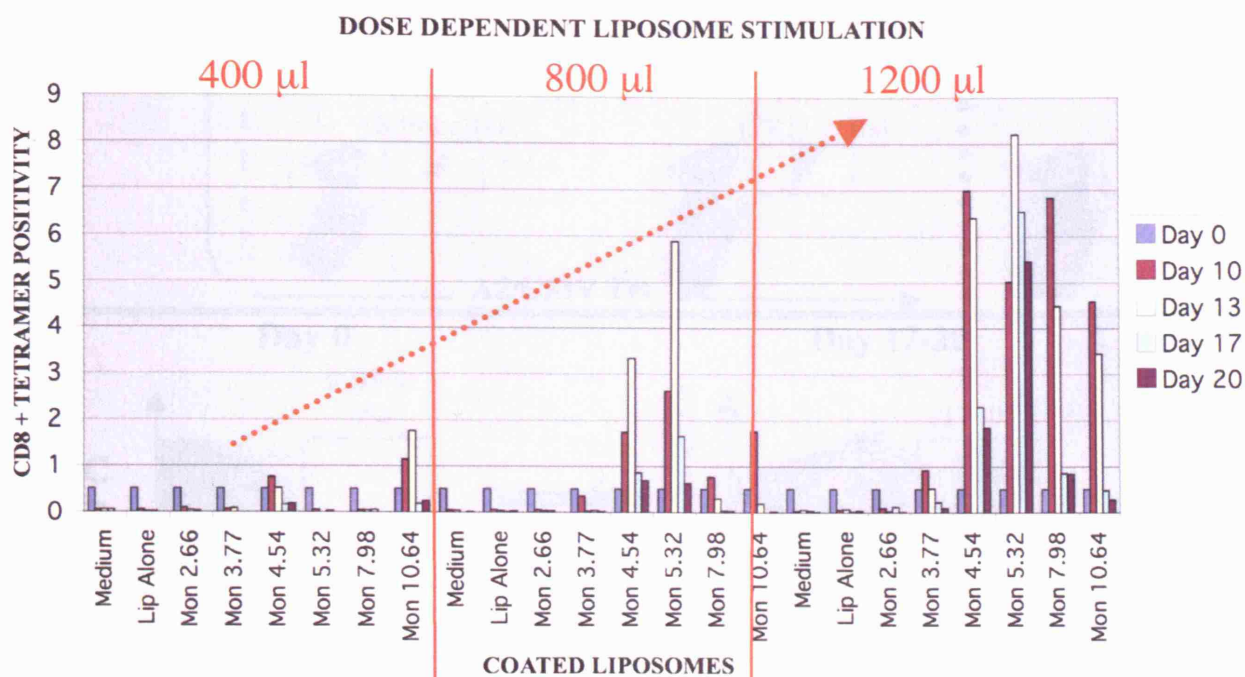


Figure 4.1.

DOSE DEPENDENT LIPOSOME / T CELL EXPANSION: PBMCs from a CMV positive individual were stimulated with liposomes coated with different amounts of MHC peptide complexes (from 2.66 µg to 10.64 µg) in three different doses (400, 800 and 1200 µl). A dose dependent correlation was found as well as an increased expansion according to the amount of MHC monomers delivered. However even at the 1200 µl optimal dose, when a large amount of monomers were delivered (10.64 µg), over stimulation and death of T cells was observed. Therefore liposomes coated with 4.54, 5.32 and 7.98 µg at the optimal dose (1200 µl) were chosen to be used in further experiments. A dose higher than 1200 µl was not explored since cells would have been over diluted in the cultures.

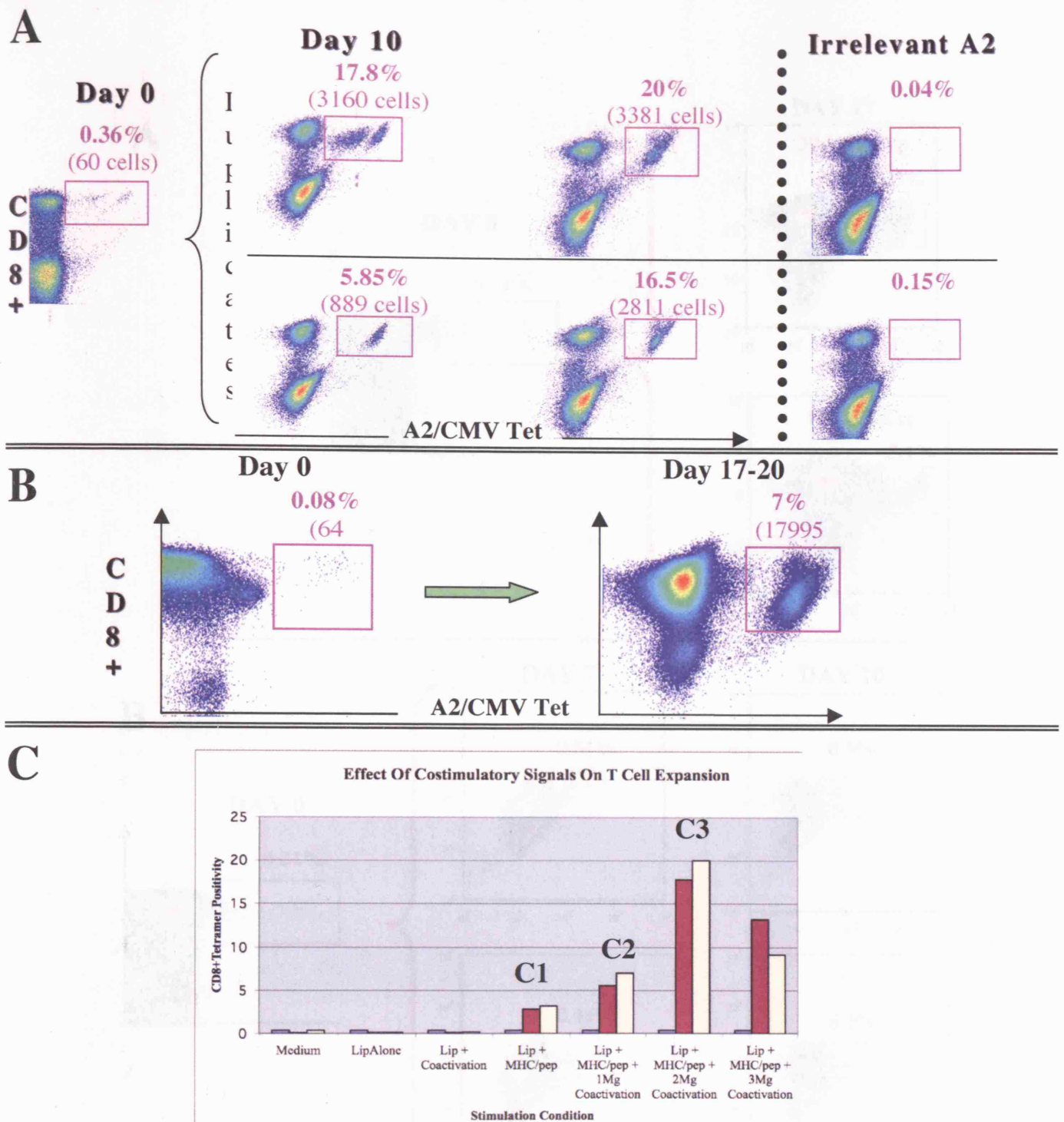


Figure 4.2.

aAPC MEDIATED T CELL EXPANSION: PBMCs (A) and purified CD8+ T cells (plus irradiated negative fraction) in B from a CMV positive individual were stimulated with the aAPCs (two rounds). C.- Shows that stimulating the cells with medium, uncoated liposomes and liposomes coated with co-activatory signals in the absence of MHC peptide complexes did not have any effect on T cell antigen specific expansion. However, an optimised number of MHC/peptide molecules on liposomes had an effect (C1), which was significantly boosted by the addition of co-activatory signals [1 μ g (C2) or 2 μ g (C3) of each one]. 3 μ g of each signal were also explored with no further effect on T cell expansion. The pink asterisk in figure A shows two separate NLV specific population, suggesting that aAPCs were able to expand different T cell clones with potentially different recognition efficiencies for the same target.

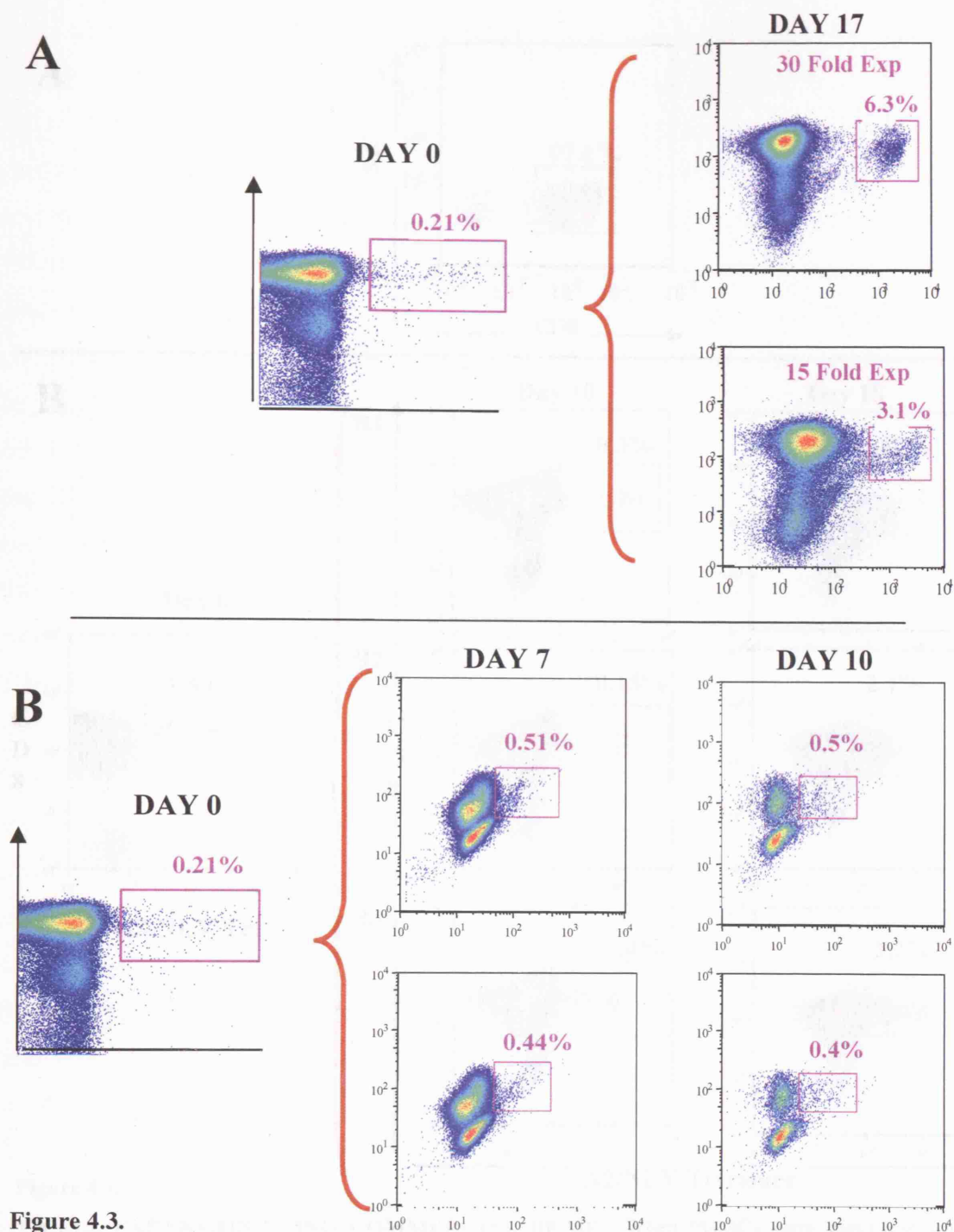


Figure 4.3.

ANTIGEN SPECIFIC T CELL EXPANSION BY DCs: **A.-** Purified CD8 positive cells from a CMV positive individual plus the irradiated negative fraction were stimulated with peptide pulsed DCs accomplishing 15 to 30 fold specific expansions. **B.-** In different cultures, the same cells were stimulated with peptide pulsed DCs plus coated liposomes. The cultures died very quickly. This is probably as a result of over stimulation that induced T cell exhaustion and apoptosis.

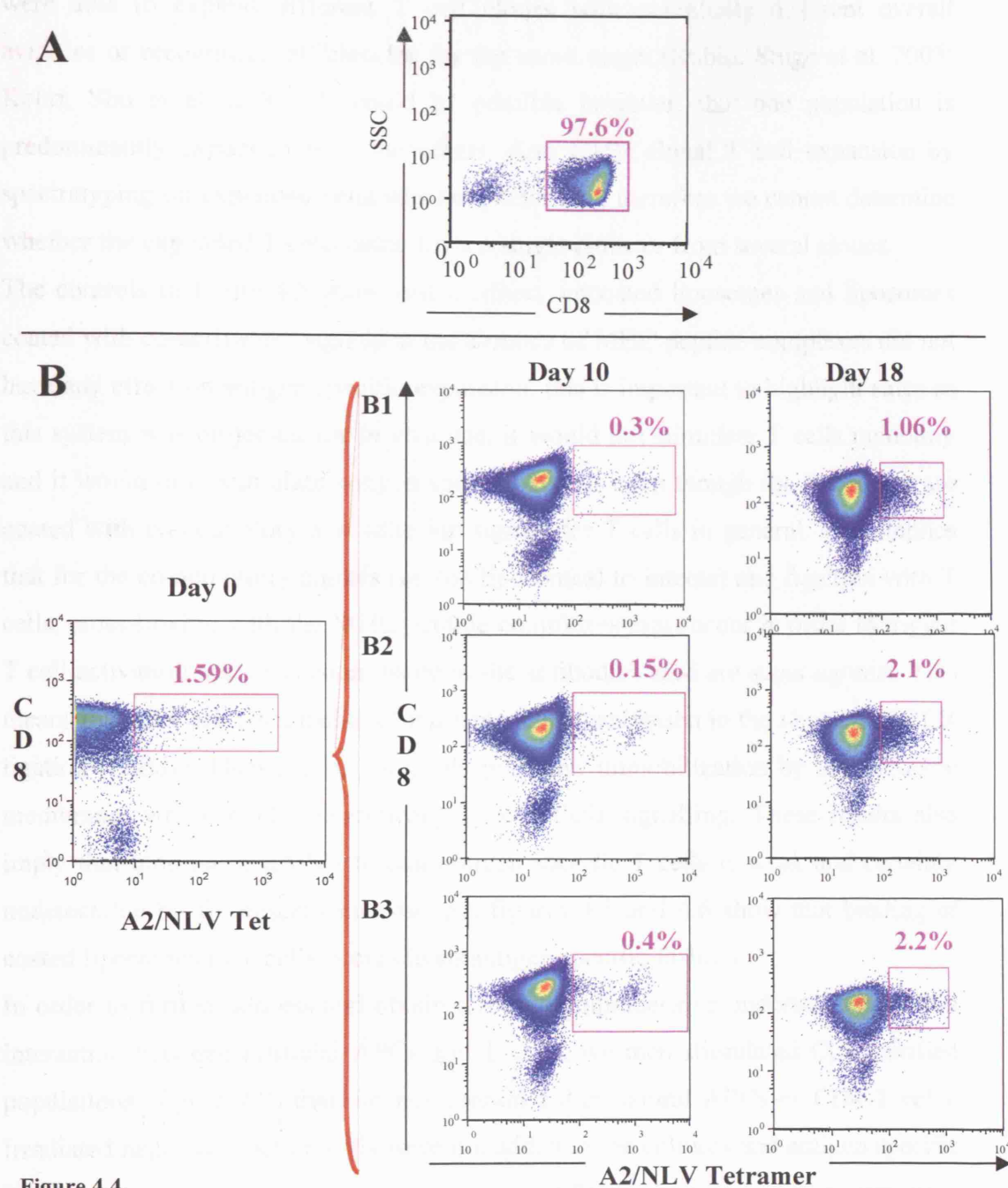


Figure 4.4.

T CELL EXPANSION USING COMMERCIAL BEADS: When PBMCs were stimulated with anti-CD3/CD28 coated commercially available beads from Dynal, CD4 polyclonal expansions are normally observed as described by Lum and LeFever et al. However when purified CD8 positive cells were stimulated (**A**). CD8s T cells including the antigen specific population shown in the pink boxes (**B1**, **B2** and **B3**) were all expanded to an extent.

were able to expand different T cell clones with potentially different overall avidities or recognition efficiencies for the same target (Rubio, Stuge et al. 2003; Kohrt, Shu et al. 2005). It could be possible however, that one population is predominantly expanded over the others. Anti CMV clonal T cell expansion by spectratyping on expanded cells was not performed, therefore we cannot determine whether the expanded T cells come from a single clone or from several clones.

The controls in figure 4.2 show that medium, uncoated liposomes and liposomes coated with co-activatory signals in the absence of MHC peptide complexes did not have any effect on antigen specific expansion, this is important to highlight since as this system was projected for *in vivo* use, it would not stimulate T cells randomly and it would only stimulate antigen specific T cells even though the liposomes are coated with co-activatory and adhesion signals for T cells in general. This implies that for the co-activatory signals (on the liposomes) to interact and function with T cells, cross-linking with the MHC peptide complexes must occur in order to trigger T cell activation and expansion. None of the antibodies used are super agonist. This means that they are not capable of inducing T cell activation in the absence of TCR ligation (Luhder, Huang et al. 2003) despite their immobilization by the liposome membrane, which could theoretically favour their signalling. These results also imply that binding of aAPCs to non antigen specific T cells is weak and certainly undetectable by fluorescent microscopy; figures 4.5 and 4.6 show that binding of coated liposomes to T cells occurs in an antigen specific fashion.

In order to further address and obtain a more comprehensive understanding of the interaction between artificial APCs and T cells, we then stimulated CD8 purified populations (figure 4.7) that did not contain either natural APCs or CD4 T cells. Irradiated negative fraction cells were not added to the cultures and antigen specific T cell expansions were still observed (figure 4.8), suggesting a direct interaction between coated liposomes and antigen specific CD8⁺ T cells as explained in detail in section 4.2.4.

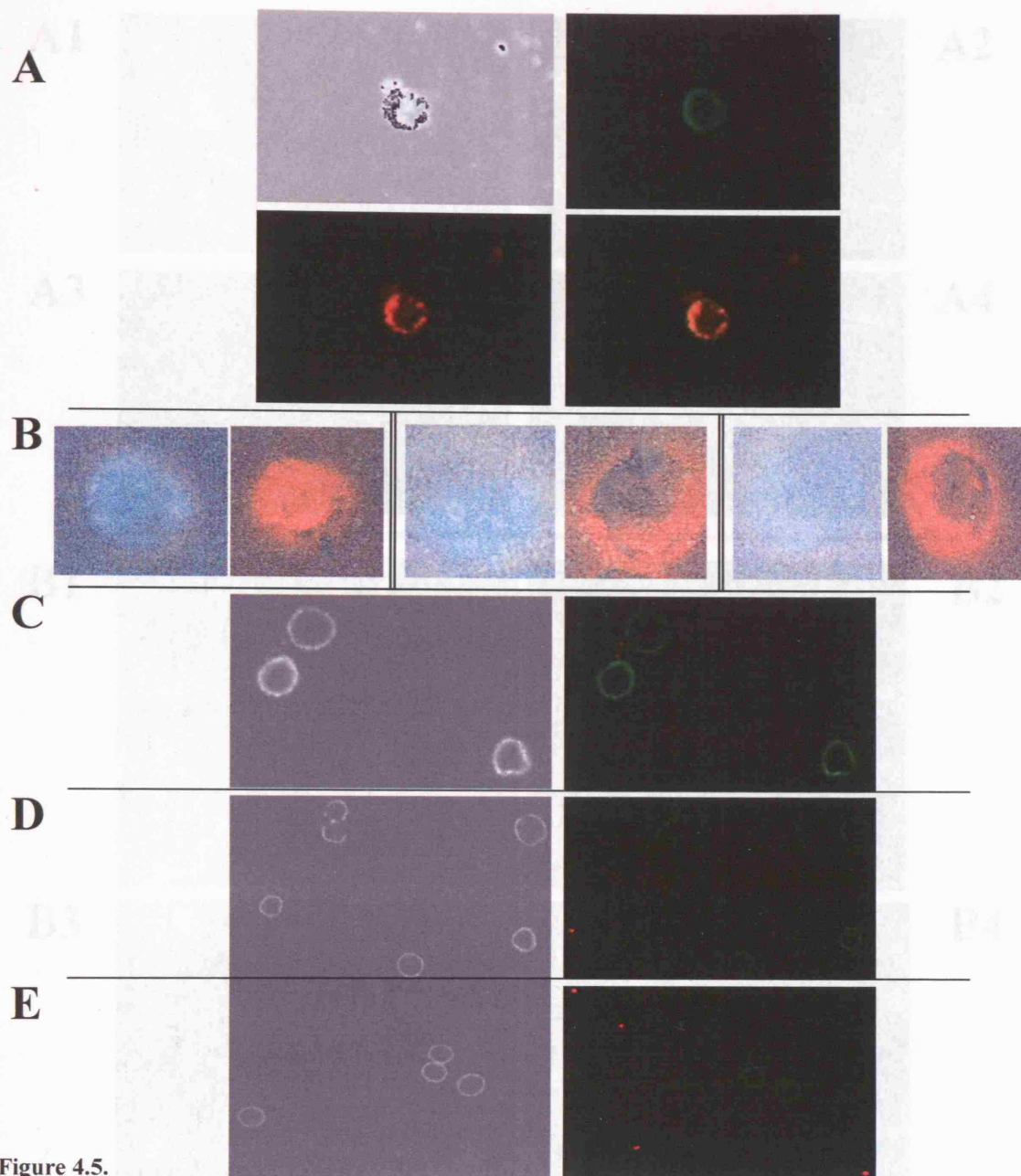


Figure 4.5.

aAPC - T CELL ANTIGEN SPECIFIC BINDING: **A.-** Shows MHC CMV peptide complex and co-activatory signal coated fluorescent liposomes in red, bound to a CD8+ T cell (stained with FITC anti CD8). **B.-** Shows three APC-CMV tetramer positive T cells in blue covered with coated liposomes showing antigen specific binding. Since uncoated liposomes do not bind to T cells (**C**) and coated liposomes did not bind to T cells in a CMV matched/HLA mismatched culture (**D**) nor in an HLA matched/CMV mismatched culture (**E**), Liposome-T cell binding was shown to be antigen specific and MHC/peptide dependent.

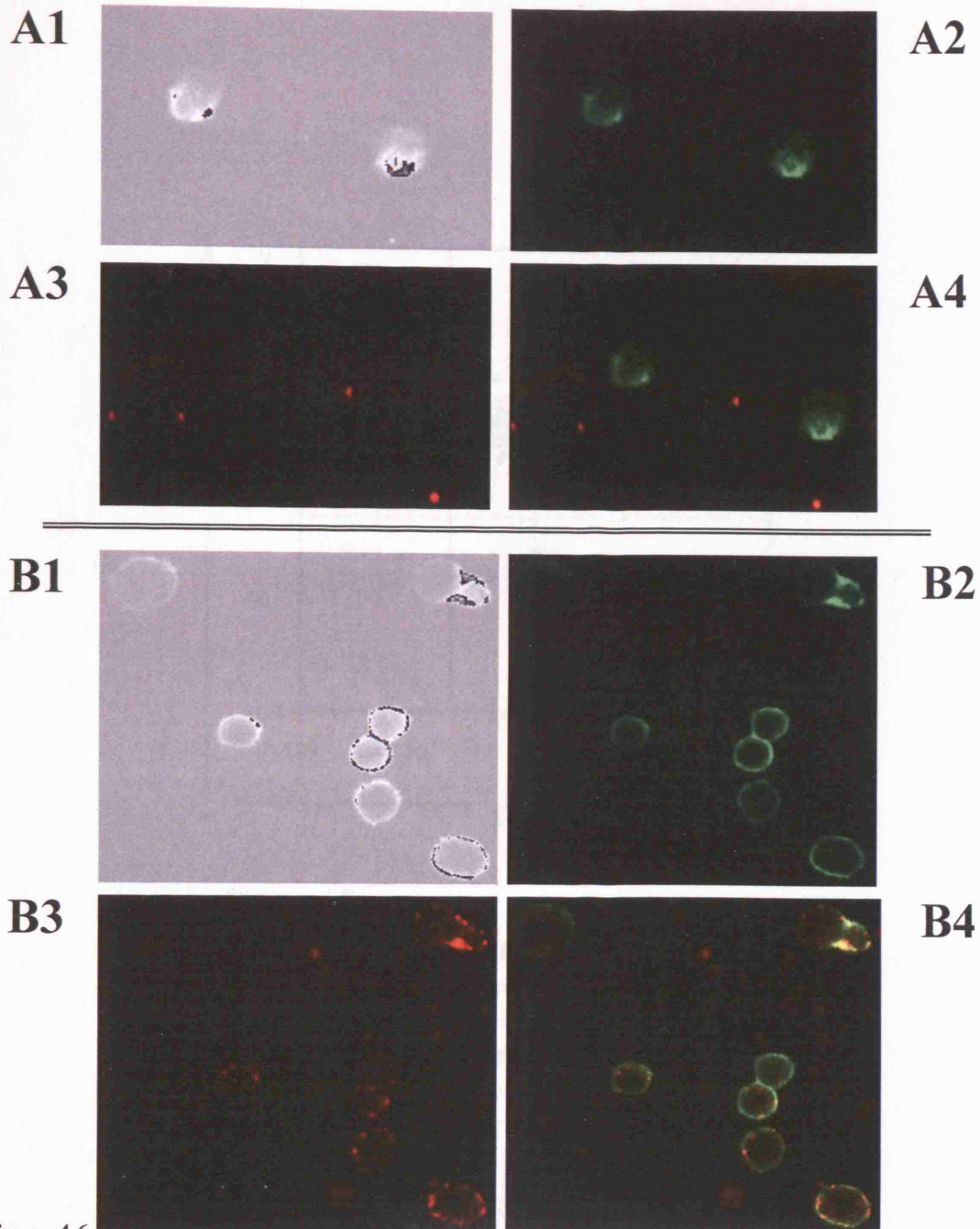


Figure 4.6.

aAPC - T CELL BINDING: A1 to A4.- MHC CMV peptide complex and co-activatory signal coated fluorescent liposomes (shown in red) in a matched HLA and CMV culture did not bind to non-specific CD8+ T cells (stained with FITC anti CD8) randomly. After antigen specific expansion however, **B1 to B4.-** many CD8+ T cells were covered with coated liposomes. **A4** and **B4** show the overlay of both colours.

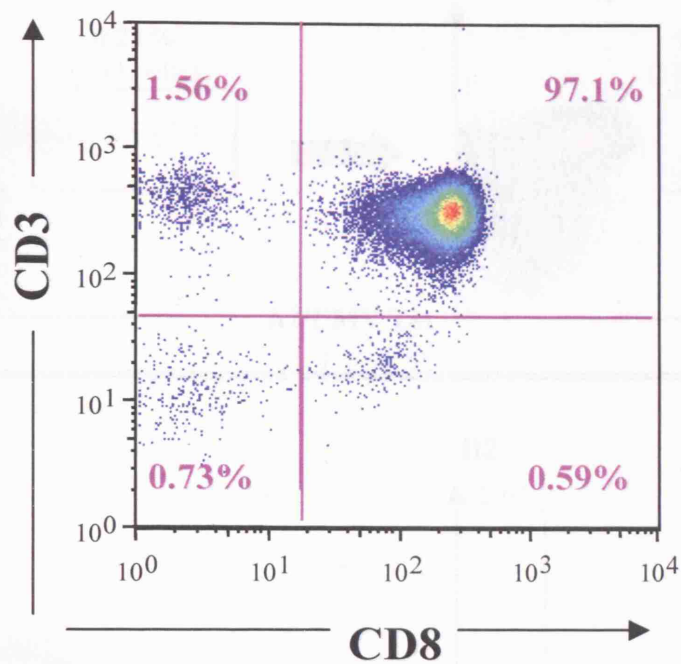


Figure 4.7.

CD8 + T CELL SELECTION: Purified CD8+ populations were isolated in order to determine the direct effect of the artificial APCs on T cells. Using anti CD8 magnetic micro beads, CD8+ T cells were isolated from the PBMC population with very high purity (~97%).

4.2.1 T Cell Activation: Expanded T Cells Vireally Reduce Degradation And Cytokine Production

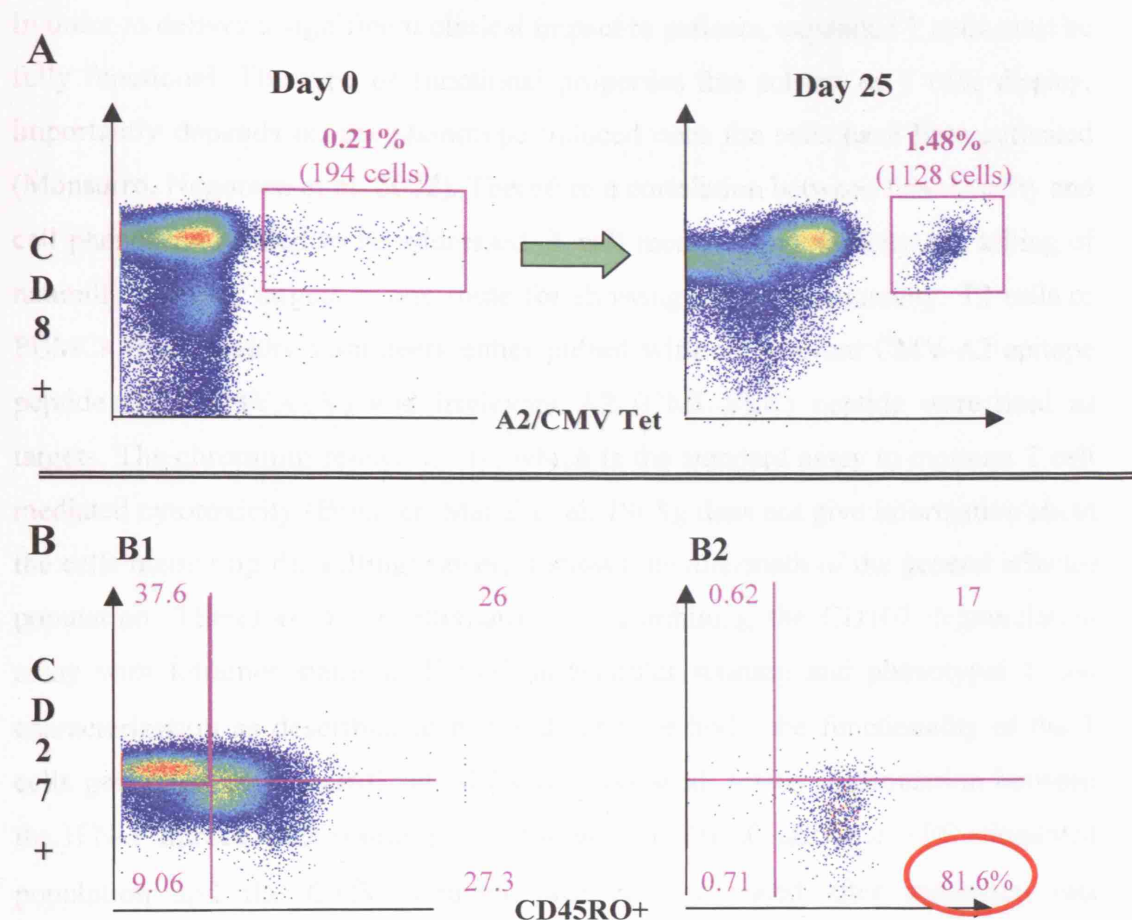
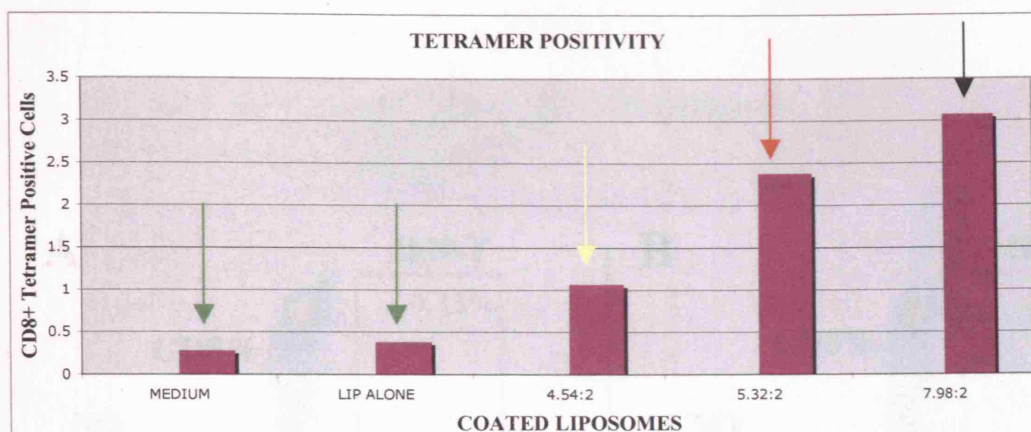
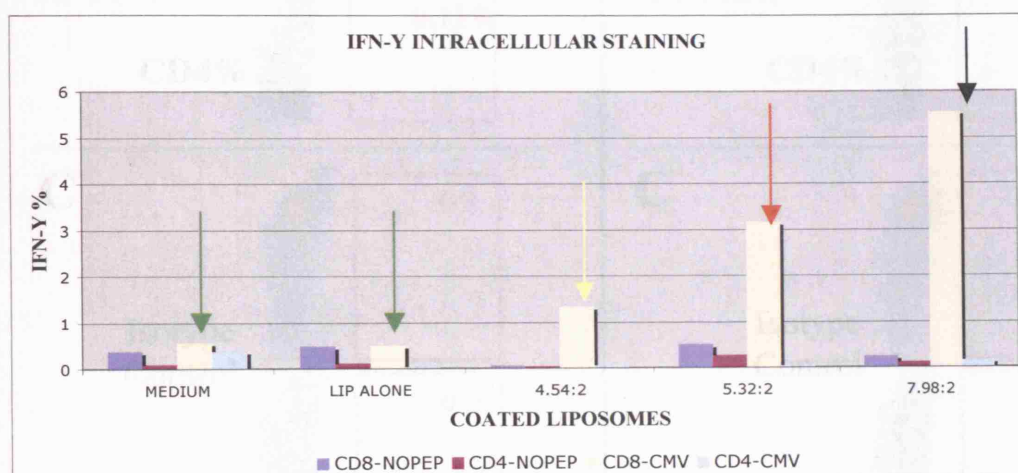


Figure 4.8.

aAPC / CD8 DIRECT INTERACTION: **A.-** Shows a 7 fold antigen specific expansion reached when purified CD8 positive cells were stimulated with the aAPCs. **B.-** Shows the phenotype of the general T cell pool (B1) and the tetramer positive cells (B2) respectively. Since no natural APCs, CD4s or irradiated feeders cell were in the cultures and since functional relevant phenotypes were preferentially induced (81.6% effector memory cells and 17% memory cells), that suggested that aAPCs were capable of triggering T cell expansion directly.

4.2.1 T Cell Function: Expanded T Cells Via aAPCs Retain Degranulation And Cytokine Production

In order to deliver a significant clinical impact to patients, expanded T cells must be fully functional. The type of functional properties that subsets of T cells display, importantly depends on the phenotype induced once the cells have been activated (Monsurro, Nagorsen et al. 2002). Therefore a correlation between functionality and cell phenotype needed to be addressed. T cell mediated cytotoxicity and killing of naturally relevant targets is one route for showing T cell functionality. T2 cells or PBMCs from healthy volunteers either pulsed with the relevant CMV-A2 epitope peptide (NLVPMVATV) and irrelevant A2 (CML-SLK) peptide were used as targets. The chromium release assay, which is the standard assay to measure T cell mediated cytotoxicity (Brunner, Mauel et al. 1968), does not give information about the cells mediating the killing; rather, it shows the aftermath of the general effector population. Therefore as an alternative, by combining the CD107 degranulation assay with tetramer staining, IFN- γ intracellular staining and phenotypic T cell characterization as described in materials and methods, the functionality of the T cells generated by the artificial APCs was assessed. Firstly a correlation between the IFN- γ intracellular staining from the general PBMC artificial APC stimulated population and the CMV tetramer positivity generated after expansion was performed. Comparison of figures 4.9A and B, shows that early following the first stimulation (day 8), only the CD8 $^{+}$ T cells produced IFN- γ when challenged with targets presenting the relevant CMV peptide (NLVPMVATV). The production of IFN- γ also correlated with the peak of the respective tetramer positive population. If there was no tetramer positivity, no IFN- γ production was detected. The higher the number of tetramer positive cells, the higher the IFN- γ production. Figure 4.10 shows a flow cytometry plot of one of the conditions from the previous experiment. Figures 4.9 and 4.10 give information on the behaviour of the CD8 $^{+}$ population. In order to look at how the tetramer positive populations were behaving, a number of functional tests were performed including tetramer staining, IFN- γ intracellular staining, T cell phenotype and CD107 degranulation assays. Figure 4.11

A**B****Figure 4.9.**

T CELL FUNCTION: CORRELATION BETWEEN TETRAMER STAINING AND IFN- γ INTRACELLULAR STAINING: PBMCs from a CMV positive individual were stimulated with medium, liposomes alone or coated liposomes with either 4.54, 5.32 or 7.98 μ g of MHC peptide complexes plus 2 μ g of each of all the adhesion and co-activatory signals. **A.-** Shows the CD8+ tetramer staining at day 8 following one round of aAPC stimulation. **B.-** Shows the IFN- γ intracellular staining at the same time point. Figures A and B symmetrically correlate. In B, only the CD8 positive populations (yellow bars) produced IFN- γ when the cells were challenged with the CMV relevant target peptide (CD8-CMV). When no tetramer positivity was detected, IFN- γ was also not produced (green arrows). Increasing tetramer positivity also shows increasing amounts of IFN- γ production (yellow, red and black arrows). In figure B, CD8-NOPEP and CD4-NOPEP describe the CD8 and CD4 response against targets loaded with an irrelevant peptide respectively, whereas CD8-CMV and CD4-CMV describe the CD8 and CD4 response against targets loaded with the NLV CMV relevant peptide respectively.

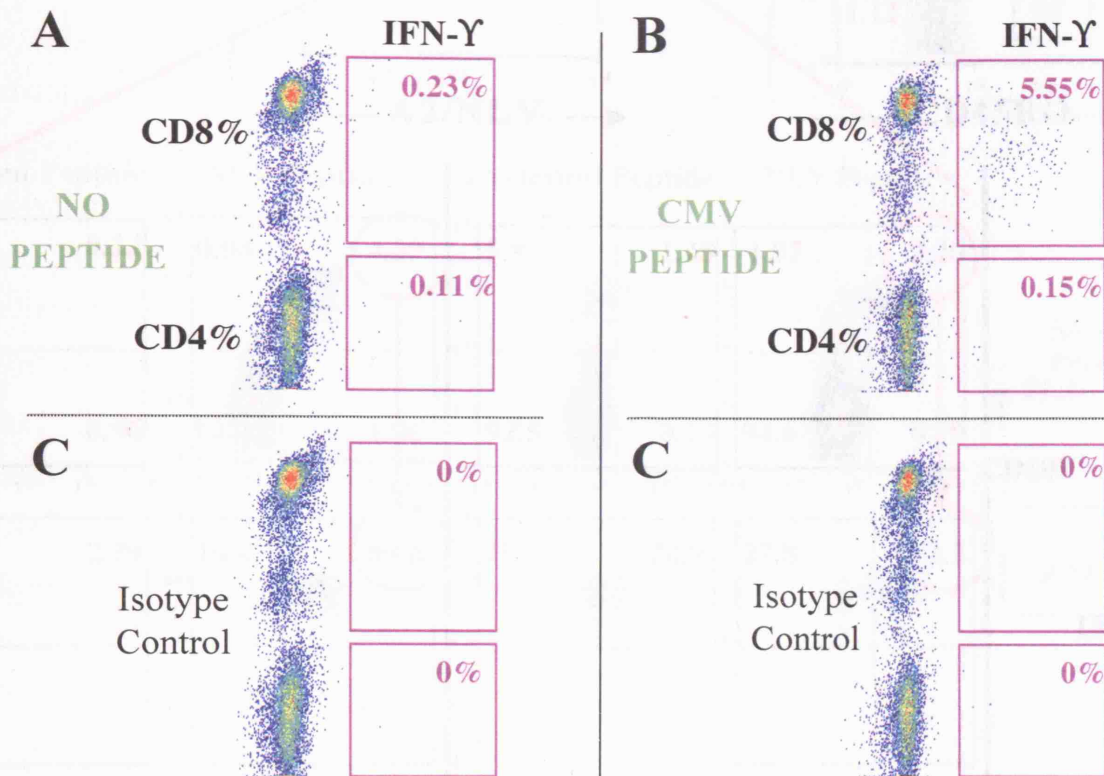


Figure 4.10.

T CELL FUNCTION: IFN- γ INTRACELLULAR STAINING: PBMCs from a CMV positive individual were challenged with PBMCs from the same individual either pulsed (**B**) or non pulsed (**A**) with the relevant NLV CMV peptide as target at day 8 after one stimulation with the aAPCs. The CD8+ tetramer positivity detected at that moment was 3.07% (not shown). **C.**- Isotype controls were also performed to compensate for background non specific staining. **A.**- Even though 3.07% of the CD8+ cells were potentially able to recognise the CMV target, when the relevant peptide was not presented there was no IFN- γ production either by CD8+ or CD4+ cells. **B.**- However when the target displayed the relevant CMV peptide, a significant percentage of CD8+ cells (5.55%) produced IFN- γ .

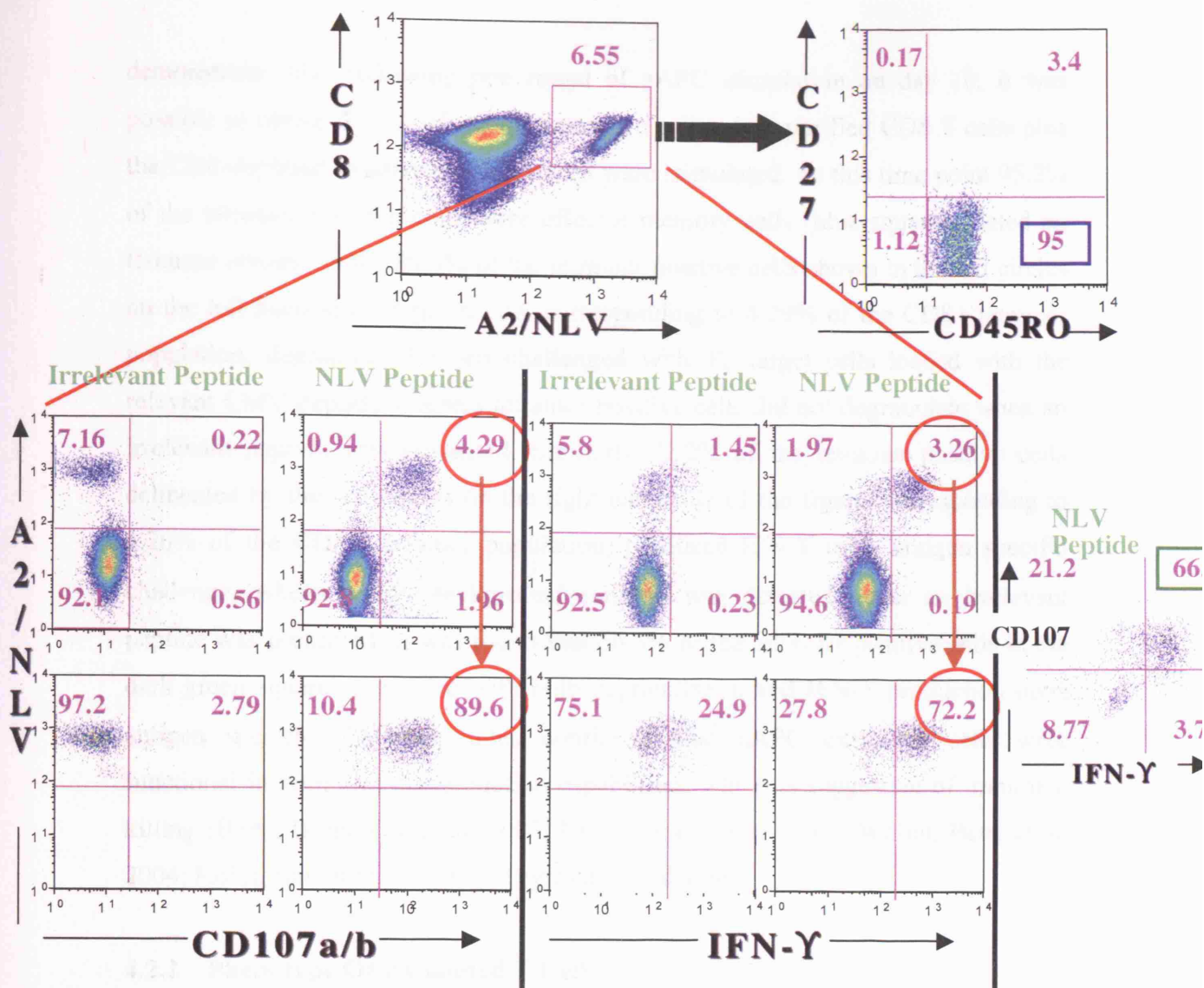


Figure 4.11.

T CELL FUNCTION: After one round of aAPC stimulation (Day 10), 6.55% of tetramer positive cells were obtained when purified CD8 T cells from a CMV positive individual (plus the negative fraction) were stimulated. At this time point 95.2% of the tetramer positive cells were effector memory (blue square). According to this and gated on tetramer positive cells, an 89.6% of the tetramer positive cells shown in the red circles on the left hand side (corresponding to 4.29% from the CD8⁺ tetramer population) degranulated when challenged with target cells loaded with the relevant CMV peptide whereas tetramer positive cells did not degranulate when an irrelevant peptide was presented. Similarly 72.2% of the tetramer positive cells shown in the red circles on the right hand side (corresponding to 3.26% from the CD8⁺ tetramer population) produced IFN-γ upon antigen specific challenge. Finally 66.3% of the tetramer positive cells (dark green square) responded with both degranulation and IFN-γ production upon antigen specific challenge. Background counts were detected with an irrelevant peptide and only showed low levels of staining. The antigen specific expansion at this time point (day 10) represents an average and expected level of expansion with the use of the artificial APCs. This figure shows that tetramer positive T cells both degranulated and produced cytokines when challenged with target T2 cells loaded with the NLV-CMV relevant peptide at 1:1 T2:CD8 T cell ratio.

demonstrates that following one round of aAPC stimulation on day 10, it was possible to obtain 6.55% of tetramer positive cells when purified CD8 T cells plus the CD8-depleted irradiated cell fraction were stimulated. At this time point 95.2% of the tetramer positive cells were effector memory cells (blue square). Gated on tetramer positive cells, 89.6% of the tetramer positive cells shown in the red circles on the left hand side of the figure, corresponding to 4.29% of the CD8⁺ tetramer population, degranulated when challenged with T2 target cells loaded with the relevant CMV peptide whereas tetramer positive cells did not degranulate when an irrelevant peptide was presented. Similarly 72.2% of the tetramer positive cells delineated by the red circles on the right hand side of the figure, corresponding to 3.26% of the CD8⁺ tetramer population, produced IFN- γ upon antigen specific challenge, whereas only background staining was detected when an irrelevant peptide was presented. It was found that 66.3% of the tetramer positive cells in the dark green square responded with both degranulation and IFN- γ production upon antigen specific challenge. This confirmed that aAPC expanded cells were functional in retaining degranulation capabilities, which is suggestive of imminent killing (Betts, Brenchley et al. 2003; Rubio, Stuge et al. 2003; Wolint, Betts et al. 2004; Kohrt, Shu et al. 2005), and cytokine production.

4.2.2 Phenotype Of Expanded T Cells

Figure 4.12 shows that following one round of aAPC stimulation on day 10, nearly 100 % of the expanded tetramer positive cells posses an effector memory phenotype. However after the second stimulation on day 13, the profile modifies and 50% of the cells remained effector memory whereas the remaining 50% were found to be only memory. CD28 and 4-1BB continuous stimulation is believed to be responsible for this switch in phenotype (Bertram, Dawicki et al. 2004; Yang, Hodge et al. 2005). It was encouraging to find that the main cell artificially APC expanded populations could be differentiated into these two main phenotypes since one of the goals of the system is to be used for both active and adoptive immunotherapy. Therefore the cells with effector memory phenotype would be able

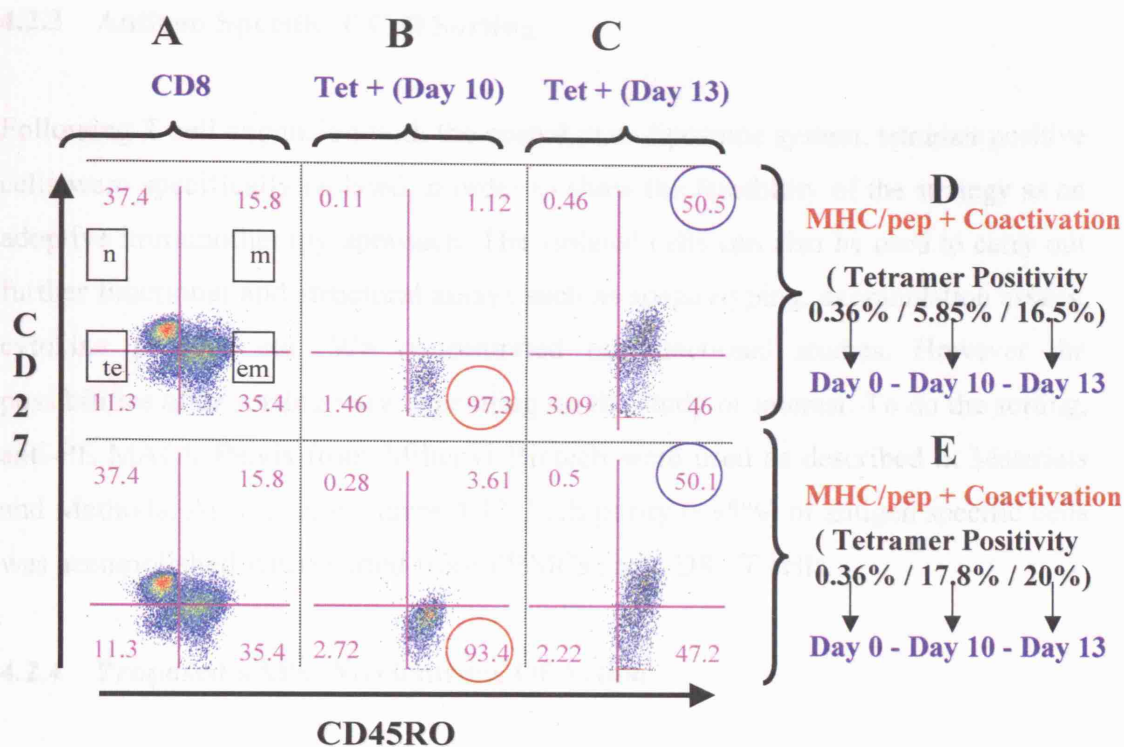


Figure 4.12.

PHENOTYPE OF aAPC EXPANDED TETRAMER POSITIVE CELLS: A.-. Shows the overall phenotype of the CD8+ population (n.-naïve; m.-memory; em.-effector memory and te.-terminal effector). B.- shows the phenotype of the expanded tetramer positive population at day 10 after one round of stimulation. C.- shows the phenotype of the expanded tetramer positive population at day 13, three days after the second round of stimulation. Two parallel expansions are shown in D and E. The behaviour of the tetramer positive population is shown below each condition. Early after the aAPC stimulation (Day 10), nearly 100 % of the expanded tetramer positive cells possess an effector memory phenotype (red ovals). However, soon after the second stimulation (Day 13), the profile modifies and 50% of the cells remained effector memory whereas the other 50% were detected as memory (blue ovals).

to provide immediate killing required to deliver immediate clinical effect in patients, whereas the cells with memory phenotype would be able to provide the potential ability to establish long-term immunological memory.

4.2.3 Antigen Specific T Cell Sorting

Following T cell expansion with the coated nano liposome system, tetramer positive cells were specifically isolated in order to show the feasibility of the strategy as an adoptive immunotherapy approach. The isolated cells can also be used to carry out further functional and structural assays such as spectratyping, degranulation assays, cytokine profiles etc. We concentrated on functional studies. However the possibilities after sorting vary depending on the study or interest. To do the sorting, anti-PE MACS Beads from Miltenyi Biotech were used as described in Materials and Methods. As shown in figure 4.13, high purity (~95%) of antigen specific cells was accomplished when sorted from PBMCs and CD8+ T cells.

4.2.4 Proposed aAPC Mechanisms Of Action

The T cell expansions shown previously could have several explanations as more than one mechanism could be operating. Firstly, coated liposomes could function directly as antigen presenting cells delivering MHC peptide complexes and co-activatory signals to T cells. A second possibility would involve the uptake of coated liposomes by natural APCs, break down of the MHC peptide complexes within the cell and cross-presentation of the NLV peptide by natural APCs using their self MHC class I molecules and co-activatory molecules. A third possibility involves incorporation and/or fusion of the coated liposome to the membranes of the natural APCs following similar kinetics as exosomes (Thery, Zitvogel et al. 2002). This would allow the coated liposomes to work in a semi-direct manner.

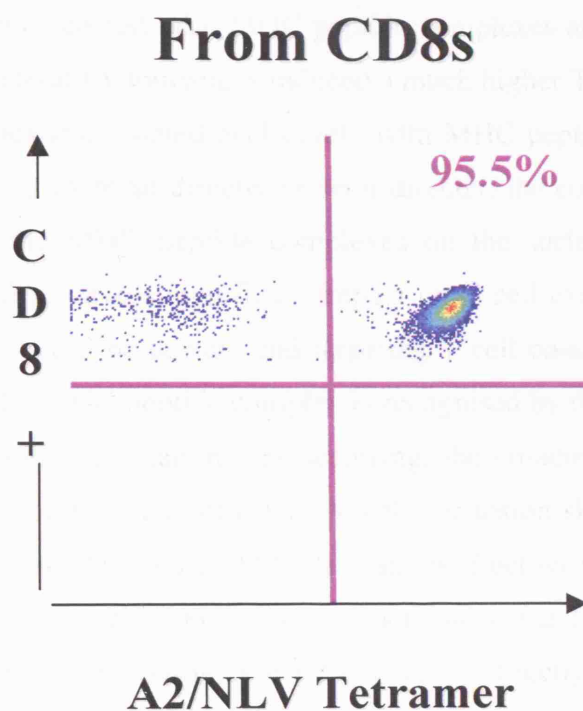


Figure 4.13 .

T CELL ANTIGEN SPECIFIC SORTING: Tetramer positive cells were isolated following expansion using the aAPCs using PE labelled tetramers and anti PE magnetic beads accomplishing 95% purity.

4.2.4.1 aAPC Direct Activation

In order to explore these hypotheses, firstly the liposomes were coated with MHC peptide complexes with or without co-activatory signals in the form of monoclonal antibodies in order to assess the overall effect on T cell expansion. Figure 4.2C shows that liposomes coated with MHC peptide complexes and well-characterised adhesion and co-activatory molecules induced a much higher T cell expansion when compared with liposomes coated exclusively with MHC peptide complexes. If the artificial APCs were working directly or semi-directly, the co-activatory signals in conjunction with the MHC peptide complexes on the surface of the liposomes would be expected to have a significant impact on T cell expansion since the co-activatory signals would be present and targeting T cell co-activatory receptors at the same time as the MHC peptide complex is recognised by the T cell receptor. On the contrary, if cross presentation was occurring, the co-activatory signals on the liposomes should not have any effect on T cell expansion since they would have been lost during uptake by natural APCs having no effect when the peptide was re-presented using the natural APC's own co-activatory machinery. Therefore our results suggested that the liposomes were interacting directly and/or semi directly with the T cells and that cross-presentation by natural APCs uptake, even though possible, was not the mode of antigen presentation when coated liposomes were in the cultures. Secondly, when CD8 purified populations with virtually no natural APCs nor CD4 T cells (fig 4.7) were then stimulated, no irradiated negative fraction was added and antigen specific T cell expansions were still observed as previously shown in figure 4.8. This level of expansion was not as high as when all populations of cells were in the culture. This served to confirm the importance of CD4 help, cytokines support produced by other cells during T cell activation and a probable synergistic effect of liposomes working as exosomes as well. However since antigen specific expansion was still observed when the artificial APCs were used to stimulate purified CD8+ populations, this suggested that the coated liposomes were working directly on T cells as micro aAPCs. Figures 4.5 and 4.6 show that binding of coated liposomes to T cells occurs only in an antigen specific fashion: figure

4.5B shows three CMV specific T cells stained with an APC-NLV pentamer in blue (kind gift from Proimmune) covered with coated liposomes, confirming their antigen specific binding. In contrast, figure 4.5C shows that uncoated liposomes do not bind to T cells non specifically and figure 4.5D shows that MHC/peptide and co-activation signal coated liposomes in a CMV matched but HLA mismatched culture did not bind to T cells in a non HLA specific manner. Fig 4.5E also shows that in a HLA matched / CMV negative situation, the coated liposomes also do not bind in a non specific fashion. Figure 4.6A demonstrates that coated liposomes (even though in a HLA matched and CMV matched culture) did not bind to all CD8⁺ T randomly. In contrast however, following T cell specific expansion using the nano liposome system, a large number of the T cells in the culture were CMV specific and as a consequence large numbers of CD8⁺ T cells were covered with liposomes (figure 4.6B). The data presented demonstrates liposome-antigen specific T cell binding. This however did not necessarily imply that liposomes were triggering activation and expansion directly. As mention previously, since purified CD8 T cells without either irradiated cells, APC or CD4 help still expanded when stimulated with coated liposomes, the antigen specific binding shown in the previous figures is believed to be sufficient to trigger T cell activation and expansion via a direct artificial APC-T cell interaction.

4.2.4.2 aAPC Semi-Direct Activation

Clearly natural APCs mount adaptive immune responses by presenting antigens to T cells in a classical and non-classical fashion. One of the non-classical pathways involving exosomes has particular relevance due to its similarity with the use of liposomes. Exosomes are involved in a semi-direct pathway of antigen presentation. They are nanoscale lipid vesicles produced by many types of cells (APCs included). Dendritic cell derived exosomes are covered with MHC class I and II peptide complexes plus some adhesion and costimulatory signals (Segura, Amigorena et al. 2005; Segura, Nicco et al. 2005). When circulating APCs capture a pathogen, they mature and travel to the draining lymph nodes. Once there, the APCs release

exosomes, which bind to other APCs in the lymph nodes. This process allows APCs that did not encounter the pathogen directly in the periphery to be primed to stimulate an immune response even when they have not directly encountered the antigens themselves (Thery, Duban et al. 2002; Thery, Zitvogel et al. 2002; Herrera, Golshayan et al. 2004). It is possible that liposomes also function in a similar manner as exosomes, by binding to natural APCs. Figure 4.14A shows that coated liposomes (red vesicles) bound as well to natural APCs (in green, stained with FITC anti DR). The figure shows a culture of APCs and CD8⁺ T cells, highlighted are three T cells (also covered with liposomes) in apparent close interaction with the APC via coated liposomes. Figure 4.14B shows that lipids themselves are the natural APCs binding target (Bendelac, Teyton et al. 2002; Moody, Briken et al. 2002; van den Elzen, Garg et al. 2005) since uncoated liposomes bind to natural APCs. All these results therefore suggest that coated liposomes activate antigen specific T cells both directly as micro aAPCs and semi-directly via natural APCs similarly to exosomes in a synergistic fashion.

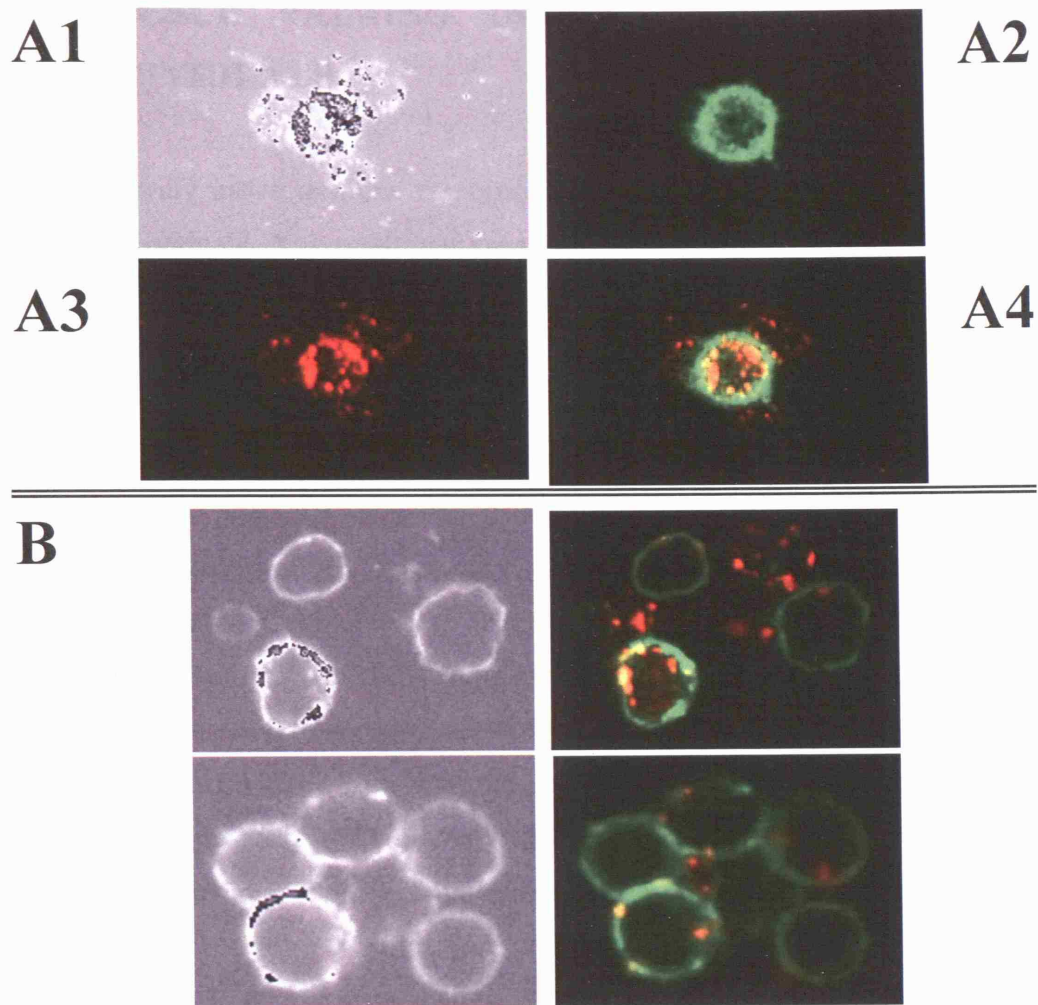


Figure 4.14.

aAPC BINDING TO NATURAL APCs: The artificial APCs bind to natural APCs in similar fashion to exosomes. **A1.-** Shows an APC surrounded by three other cells. **A2.-** Shows the central cell as a class II positive cell staining (in green) with FITC anti DR antibodies. **A3.-** Shows the same cell covered with red fluorescent coated liposomes. **A4.-** Overlay of both colours. The three surrounding cells are CD8 T cells as only monocyte DCs and positive selected CD8s were co-cultured. The T cells were also covered with red coated liposomes. This figure shows an interaction between T cells and natural APCs where coated liposomes seem to be also involved. **B.-** Shows the spontaneous binding of uncoated liposomes to natural APCs suggesting that lipids themselves are the binding target.

4.3 T CELL PRIMING IN NAIVE (CMV NEGATIVE) INDIVIDUALS

Primary or truly naïve immune responses have different kinetics to those seen in memory individuals. Naïve antigen specific T cell precursors are extremely low, with levels reported to be one T cell precursor in 1 million circulating T cells (Rouse, Larsen et al. 1983; Posavad, Koelle et al. 1996; Blattman, Antia et al. 2002). This is one of the reasons that naïve responses are much more difficult to mount *ex vivo*. As discussed previously, memory T cells can be expanded by MHC peptide complex engagement in the absence of co-activatory signals (figure 4.1). For naïve T cells however, a selection of adhesion, early and late co-activatory signals are essential and required to accomplish naïve T cell activation. That is one of the reasons that only dendritic cells are considered capable of stimulating such responses. When naïve responses have been reported *ex vivo*, much lower tetramer positive populations have been reported and even so, it is critical to show that the population is truly derived from a naïve precursor since often the expansion can be the result of a memory cross-reactive low frequency clone, which can go undetected as background by tetramers before stimulation.

For memory T cells, the role that each co-activatory signal possesses has been better established. Unfortunately that is not the case for naïve T cells. Some reports suggest that CD27 might be more important than CD28 and vice versa (Watts and DeBenedette 1999; Croft 2003; Hendriks, Xiao et al. 2003). CD40 ligand has been shown to be essential as well as the presence of the LFA-1 / ICAM-1 adhesion receptors. Taking all these factors into account, we took a different approach during the stimulation of naïve samples. In order to explore this, liposomes tested in the naïve setting were constructed in several different MHC peptide/co-activatory ratios and combinations using only a selection of secondary signals. Having standardised the stimulation conditions in the memory setting, it was decided to use the same number of cells and the same protocol timetable at first. PBMCs (2 million) were stimulated in each culture and the populations detected are shown in figure 4.15. We felt that perhaps two million PBMCs might have been an under-estimation due

to the low frequencies of naïve T cell precursors as previously mentioned. However, even with these low numbers of stimulated cells, CD27 seemed to induce better naïve T cell activation than CD28. Liposomes coated only with monomers had no influence for priming, which confirmed the fact that co-activatory signals are essential to prime naïve immune responses. As shown in figure 4.15B and C, a significant level of background was detected in the flow cytometry plots (blue squares). However these background populations were still less than half of the tetramer positive populations when specifically gated. In order to address whether these tetramer positive populations were real, functionality needed to be addressed. Unfortunately cell functionality could not be performed since IFN- γ intracellular staining simply cannot be detected with such small tetramer positive numbers. It is important to mention that no adjuvants or danger signals were used to stimulate naïve T cell responses as reported by other groups i.e. α -galactosylceramide and poly I:C (Kitamura, Iwakabe et al. 1999; Rouas, Lewalle et al. 2004). These experiments were repeated adding anti CD40 ligand to the liposomes (figure 4.16) and higher populations were detected, suggestive of CD4 T cell recruitment to the immunological synapse during T cell activation, which is critical. Due to low cell numbers functionality of cells could not be addressed. Much of the work was focused on establishing parameters to the memory setting with the aim of defining the stimulation conditions on a well-established system. The naïve setting was then explored subsequently. After realising however that the naïve setting required as much standardisation as the memory setting, many optimal conditions could not be fully explored in naïve individuals. However promising results were generated on naïve samples using the aAPCs generating what appears to be an initial level of successful T cell priming. Continuation of this work is a priority and will be performed at the Anthony Nolan following this PhD.

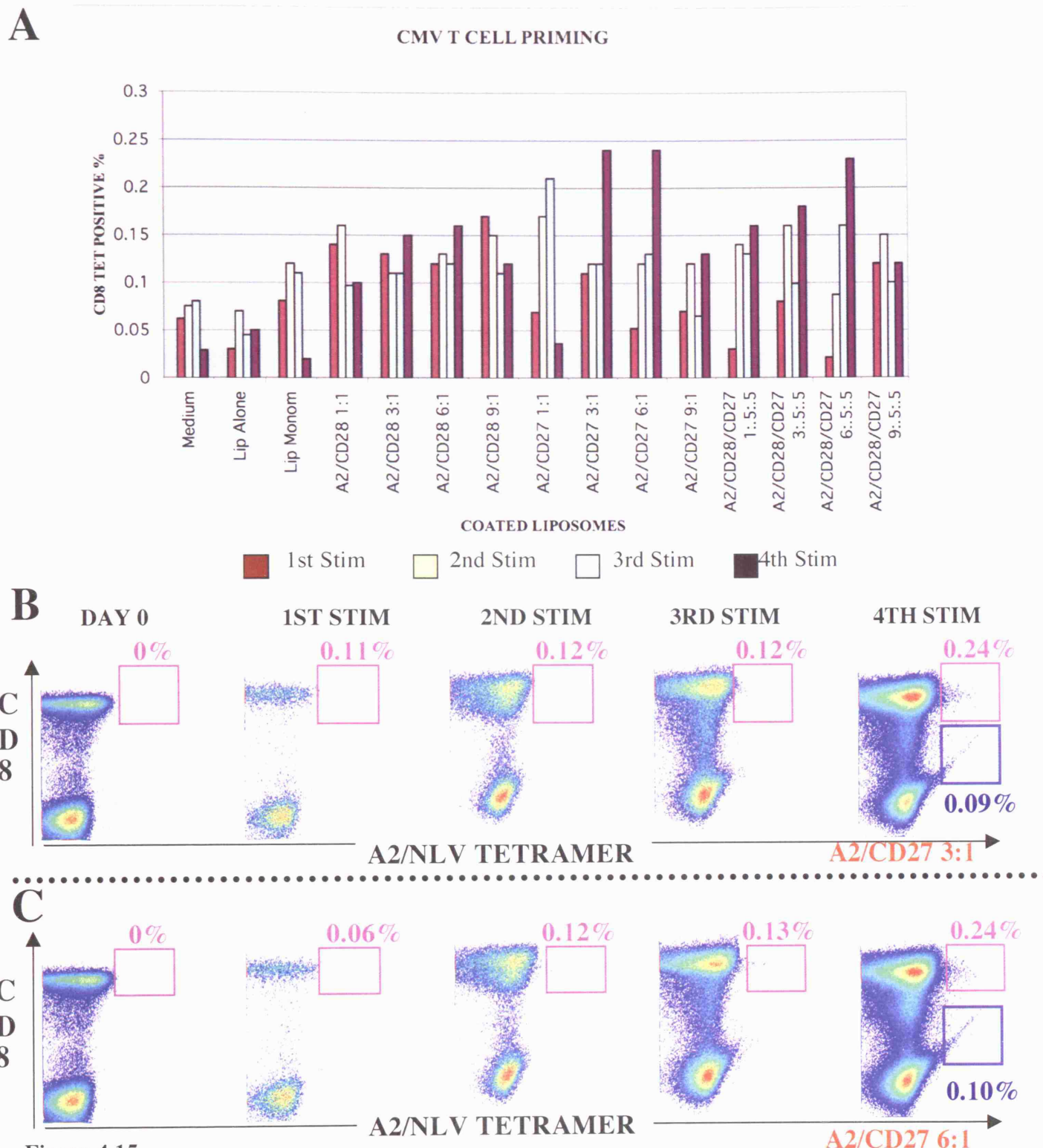


Figure 4.15.

T CELL PRIMING IN NAÏVE CMV NEGATIVE INDIVIDUALS: A.- PBMCs from a CMV negative healthy individual were stimulated with coated and uncoated liposomes as aAPCs four times in 5 day intervals. Different MHC monomers : costimulatory signals ratios and combinations were examined as shown in A (in black) and in red in B (A2 CMV MHC peptide complexes / CD27 3:1 and 6:1 ratio). **B** and **C**.- Show two (parallel) of the highest priming inductions in this experiment according to the CD8+ tetramer positivity (pink squares). The blues squares represent background.

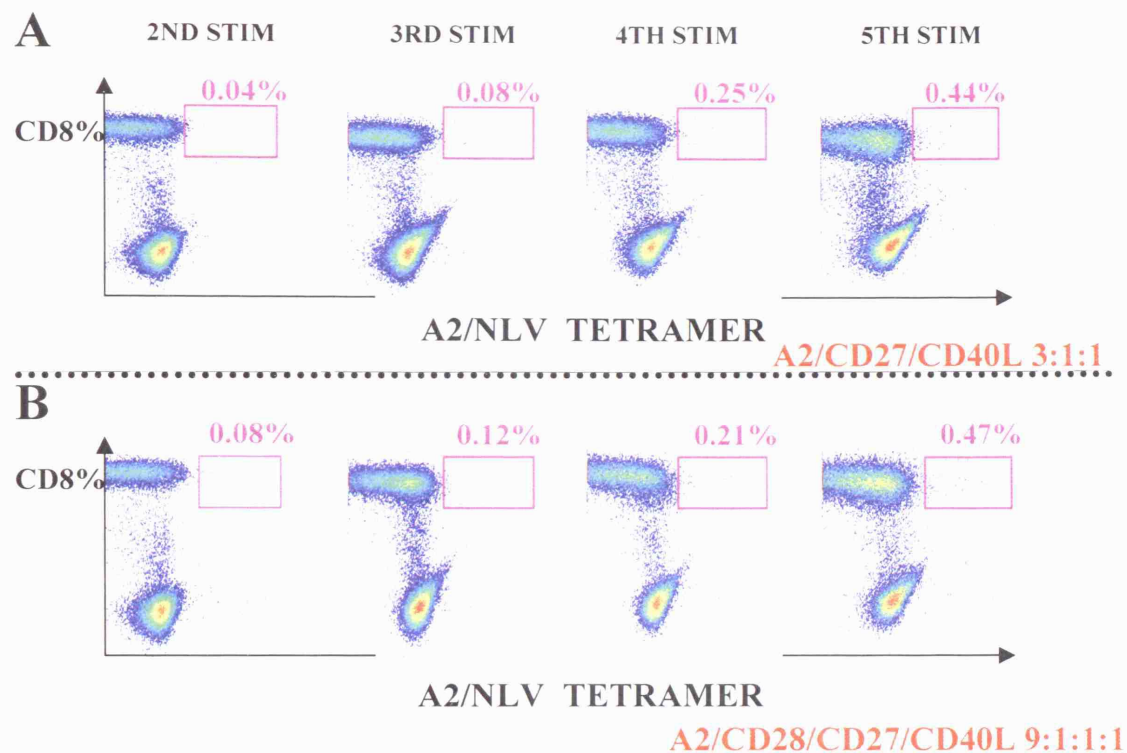


Figure 4.16.

T CELL PRIMING IN THE PRESENCE OF ANTI CD40L: PBMCs from a CMV negative individual were stimulated with coated liposomes as aAPCs with the corresponding co-activatory ligands shown in red. The tetramer plots from day zero and 1st stimulation are not shown since the percentages were undetectable. **A** and **B**.- Show two of the highest priming inductions in these experiments according to the CD8⁺ tetramer positivity (pink squares). Anti CD40L seemed to induce a higher impact on the induction of naïve responses.

4.4 DISCUSSION

Since coated liposomes have been produced specially for targeting antigen specific T cells as they have been coated with MHC/peptide complexes and T cell co-activatory signals, coated liposomes bind directly to antigen specific T cells. The ligands on T cells are the T cell receptor (TCR) and the receptors LFA-1 for the anti LFA-1 antibody, CD28 for the anti CD28, CD27 for the anti CD27, CD40L for the anti CD40L and 4-1BB for the anti CD4-1BB antibody. In contrast the binding target for natural APCs are the lipids in the liposomes. These were the two main mechanisms found to be responsible for the T cell expansions and interactions previously shown. However a number of other possibilities exist for the expansions observed and here will be discussed. Stimulation of purified CD8 populations (fig 4.7 and 4.8) could result in antigen presentation mediated by T cells themselves in a T cell - T cell antigen presentation manner. In order for T cell - T cell antigen presentation to occur, a number of simultaneous events are required. One is that when coated liposomes bind to T cells (figure 4.5A), the T cell membrane (covered with the MHC peptide complexes and co-activatory signals from the liposomes) could act as an APC and that could potentially allow T cell - T cell antigen presentation, which is still artificial APC mediated: however it could allow T cell - T cell antigen presentation. Another mechanism for T cell - T cell antigen presentation could be mediated by recombinant monomer-peptide unfolding, the free peptide could compete with low affinity peptides on the surface of the T cells, they could therefore "re-present" the peptide and that could also potentially explain T cell - T cell antigen presentation. This mechanism seems very unlikely since liposomes themselves and the link between them and MHC peptide complexes is very strong and stable as previously mentioned. Whatever the case however, when T cell - T cell antigen presentation occurs, T cells do not get expanded, instead T cell fratricide occurs and T cell - T cell killing dominates (Trambas and Griffiths 2003). Therefore the T cell antigen specific expansion observed in our cultures after purified CD8 populations were stimulated, was believed to be the result of coated liposomes working directly as aAPC rather than T cell - T cell antigen presentation.

As another potential aAPC mechanism of action, figure 4.14 shows natural APCs covered with coated and uncoated liposomes. Consequently liposomes could be eventually internalised and cross presentation (Belz, Carbone et al. 2002) by natural APCs could take place. This possibility was important to address since if found positive, it would not be any advantage in coating liposomes with co-activatory signal and recombinant MHC/peptide complexes as they would be lost during cross-presentation and would have no effect on T cell activation since natural APCs would only use the peptide using both self MHC and self co-activatory signals. Cross presentation was found not to be an impediment. We were able to address this first because the standard naturally occurring APC population sorted by PBMCs purification, is not sufficient to stimulate the level of expansion that we have observed (200 fold). That is exactly why in order to expand T cells using dendritic cells, they have to be grown, matured and generated first from monocytes recovered from PBMCs in order to have sufficient numbers and proper functional capabilities. Secondly, as shown in figure 4.2C, the level of T cell specific expansion was at least doubled when co-activatory signals were also delivered along with the MHC peptide complexes on the surface of liposomes, suggesting that these co-activatory signals were directly triggering T cell activation when delivered onto the liposomes in the presence of signal one. If cross-presentation was occurring, the co-activatory signals should not have had an important effect since they would have been lost during APC internalization. Finally figure 4.8 showed that liposomes were sufficient to expand purified CD8 positive T cells when virtually no natural APCs were in the culture, which demonstrates that the artificial APCs were directly presenting the specific peptide and triggering activation and expansion. Therefore even though cross presentation could potentially take place, it is not the main pathway of antigen presentation and T cell activation when coated liposomes were present in the cultures.

Even though the data obtained was encouraging, a number of questions still remain. In any adaptive immune response, CD4 T cell help plays an indisputable role. They deliver signals directly to CD8s (IL-2 for example). They deliver signals indirectly to CD8s for survival and for complete and full activation via DCs by means of the

CD40-CD40 ligand pathway. They also activate B cells for antibody production and are able to kill relevant target cells directly (Thomas and Hersey 1998). CD4 T cell killing is often under-estimated in the immune response but plays an important role in adaptive immune responses (Yanai, Ishii et al. 2003). Therefore class II liposomes should be generated in order to stimulate both the CD8 and the CD4 branches of the adaptive immune response. We do have an in-house class I monomer and tetramer construction facility and well-standardised protocols. Unfortunately we do not have the same facility for class II constructs. Class II monomers can be purchased, but their cost is still prohibitive for the amount of experiments that need to be performed. However liposomes for class II activation must be eventually produced in order to deliver a complete and successful immune response.

It is important to highlight the fact that even though functional and high numbers of antigen specific T cells can be successfully artificially expanded. That does not necessarily mean that tumour or viral tolerance is going to be broken. In other words, the fact that fully functional T cells can be effectively artificially activated and expanded, does not mean that these T cells will kill and eradicate the infected or tumour cells. This is because target cells can be protected by regulatory T cells (Kono, Kawaida et al. 2005; Valzasina, Piconese et al. 2006), can lose or down regulate MHC-peptide complexes (Garrido, Ruiz-Cabello et al. 1997; Garrido and Algarra 2001), mutate or change the target peptide or secrete T cell suppressive cytokines (Cortes, Talpaz et al. 1995; Krasagakis, Tholke et al. 1998) making a fully activated T cell into a completely unresponsive one. These obstacles must be blocked and/or overcome in order to facilitate regression. Nevertheless when tumour and pathogen tolerance is indeed broken, either antigen presentation has been successfully by-passed or perfect antigen presentation has been accomplished. That is therefore the importance of creating a more comprehensive, reliable, superior and complete artificial APC as possible.

It has been discussed previously that cells do not respond well to *ex vivo* manipulation and that long T cell cultures affect T cell *in vivo* survival following infusion. That is one of the main reasons for the search of artificial systems capable

of activating and expanding T cells *in vivo*. However and even though artificial APCs for *in vivo* use are incredibly exciting since treatment should become easier and more physiological, there is a scenario where *in vivo* aAPC systems might have limited use. Sometimes the T cell clones or the T cell precursors that could be potentially activated within the human body have been already deleted, killed or anergised from the repertoire as a consequence of the immune evasion and suppression tumour or viral strategies discussed previously. In these cases, the target T cells are no longer available for activation or expansion. However this flexible aAPC system will also allow the *ex vivo* expansion of T cells from donors, specifically collected and adoptively infused into the original patient.

4.5 CONCLUSIONS

A novel artificial antigen presenting cell has been developed aiming to be used for both active and adoptive immunotherapy. The nano artificial antigen presenting cells were shown to generate 55 to 200 fold reproducible antigen specific T cell *ex vivo* expansions in CMV positive individuals. The level of expansion reached by the system was superior to those accomplished by the use of dendritic cells, which is the standard current approach and other artificial APC systems currently tried at the Anthony Nolan Research Institute. Expanded T cells are functional in terms of degranulation and cytokine production after antigen specific challenge. Following one round of stimulation, expanded T cells expressed mainly effector-memory phenotype. Following two rounds of stimulation however, 50% of the T cells generated expressed a memory phenotype whereas the remaining 50% percentage expressed an effector-memory phenotype. These phenotypes should theoretically provide enough T cells to eradicate targets in the short-term (effector-memory cells) and enough T cells to deliver immunological memory in the long-term (memory cells). Antigen specific T cells following expansion were also shown to be successfully isolated with high purity in case that adoptive immunotherapy was the aim. With the use of fluorescent aAPCs and different T cell cultures, it was shown that the aAPCs bind and activate antigen specific T cells both directly functioning

as micro aAPCs and semi-directly via APCs in similar fashion to exosomes. Cross presentation (even though possible) was found not to be a mechanism responsible for the expansion. Regarding the naïve setting, an initial level of successful T cell priming appears to have been generated even without the optimal conditions. Hopefully the system will be used as a new platform for active and adoptive immunotherapy in the near future.

CHAPTER 5

aAPC FOR ACTIVE IMMUNOTHERAPY

5.1 INTRODUCTION

Active immunotherapy as antigen specific T cell *in vivo* activation is theoretically more ideal when compared to adoptive immunotherapy (T cell *ex vivo* activation) for a number of reasons: Firstly T cell *in vivo* activation and expansion avoids culturing T cells, which is one of the most critical and important differences since T cell *ex vivo* manipulation affects T cell viability and *in vivo* survival after infusion (Dudley and Rosenberg 2003). It also avoids the use of closed systems during the manipulation and infusion of the therapeutic cells, which possess a potential risk of infection and/or contamination. Another important factor is that active immunotherapy might importantly reduce the risk of inducing graft versus host disease (GvHD) since endogenous cells from the patient are activated rather than cells coming from donors, which might have different levels of HLA histocompatibility. Having said this, adoptive immunotherapy could also expand T cells (*ex-vivo*) from the patients themselves in order to avoid GvHD, however that is not always possible due to the poor general conditions of the patients and difficulties to bleed and obtain good quality cells. As previously mentioned however, even when cells from patients are *ex vivo* manipulated, the quality, viability and survival capabilities of the cells could be compromised during the process.

It is well accepted that active immunotherapy produces a more physiological T cell expansion. However it is not always ideal since active immunotherapy also possesses risks. Firstly it is of paramount importance to ensure that T cell *in vivo* activation will occur in an antigen specific fashion. Otherwise random and general T cell activation can induce cross reactivity, autoimmunity, cytokine storms and damage to the patients, which is potentially irreversible. This is not the only

concern with active immunotherapy, several active immunotherapy clinical trials have resulted in unfortunate experiences (Lee, Wang et al. 1999; Pinilla-Ibarz, Cathcart et al. 2000; Cathcart, Pinilla-Ibarz et al. 2004). As examples of these, peptides for *in-vivo* DC mediated T cell activation have been given without the correct danger signals or adjuvants, as a consequence regulatory-DCs rather than activatory-DCs were generated (Mahnke, Knop et al. 2003) and different scenarios were observed. The consequences of such manipulations have been the induction of regulatory T cells, T cells precursor deletion and anergy induction in memory T cells (Hawiger, Inaba et al. 2001). As a result the overall effect is that tumour tolerance is boosted and the patients not only do not improve, but on the contrary worsen. Following these experiences, many of the active immunotherapy approaches have therefore concentrated on the correct and full maturation and activation of DCs and T cells in order to mount effective immune anti-tumour responses. *Ex vivo* manipulation and full *ex vivo* activation-maturation of DCs can overcome some of the immune evasion and suppression mechanisms described previously in the introduction. These *ex vivo* matured DCs can efficiently and effectively activate and expand specific T cells in both *in vivo* (Lodge, Jones et al. 2000; Lau, Wang et al. 2001; Su, Peluso et al. 2002; Bozza, Perruccio et al. 2003) and in *ex vivo* settings (Choudhury, Liang et al. 1999). Unfortunately, even though the use of dendritic cells is one of the best currently available standard methods to expand T cells, the main drawbacks of using DCs for these purposes include the amount of blood required to obtain sufficient numbers of cells, the expense of the process due to the high concentration of cytokine cocktails required, the time consuming nature of the protocol, the lack of reproducibility among samples and the necessary manipulation of DCs themselves which is not ideal. Therefore in order to avoid *ex vivo* DC manipulation, strategies to manipulate DCs *in vivo* have been initiated. Vectors carrying peptide or costimulatory molecule DNA sequences for *in vivo* DC transfection have been described (Mackensen, Herbst et al. 2000; Cerundolo, Hermans et al. 2004). Even though this branch of gene therapy is exciting and promising, it possesses all the drawbacks and fears of *in vivo* human gene transfection such as biosafety (Hacein-Bey-Abina, von Kalle et al. 2003),

tumourogenicity and engraftment. Dendritic cells are not easy cells to transfect and when successful, transfection is not long lasting due to mechanisms that are not currently well defined. This has therefore added impetus to the drive for developing and improving artificial systems to accomplish antigen presentation for immunotherapy in alternative safer and more specific ways. Even when *in vivo* antigen presentation is delivered in an effective manner, active immunotherapy could still face a compromise in that the T cells that could be potentially *in vivo* activated may not be available any longer through deletion or by been anergised by immune evasion and suppression mechanisms such as immunosuppressive cytokines, regulatory DCs and regulatory T cells amongst other factors. These mechanisms could be in part responsible for the onset of the disease itself in the first instance and unfortunately active immunotherapy may not be an option in these situations. Therefore sometimes the only alternative is returning to donors in order to develop an adoptive immunotherapy regime to deliver a clinical effect in the patient. Ideally and hopefully, the novel artificial nano system proposed here and generated during this PhD project will offer a flexible system for both active and adoptive immunotherapy depending on the unique and particular needs of the patients.

The *ex vivo* data presented in chapter 4 showed reproducible and functional antigen specific T cell expansions superior to other systems in CMV positive individuals using coated immuno liposomes as artificial APCs. Adoptive immunotherapy will hopefully form part of the future for this approach. However active immunotherapy, when possible as outlined previously, offers treatment simplicity and more potential benefits for the patients. Therefore active immunotherapy would also be an ideal achievement for the aAPC system. It is important to remember that these aAPCs are a non-cell based system (figure 5.1). They enter the body as “already mature dendritic cells” that do not require further maturation or peptide processing and loading. Therefore they cannot be affected by many of the immuno suppressive cytokines produced by tumours, pathogenes, regulatory dendritic cells or regulatory T cells. These artificial APCs are not targets for viral infection, which induce peptide-MHC unsuccessful loading and MHC down regulation

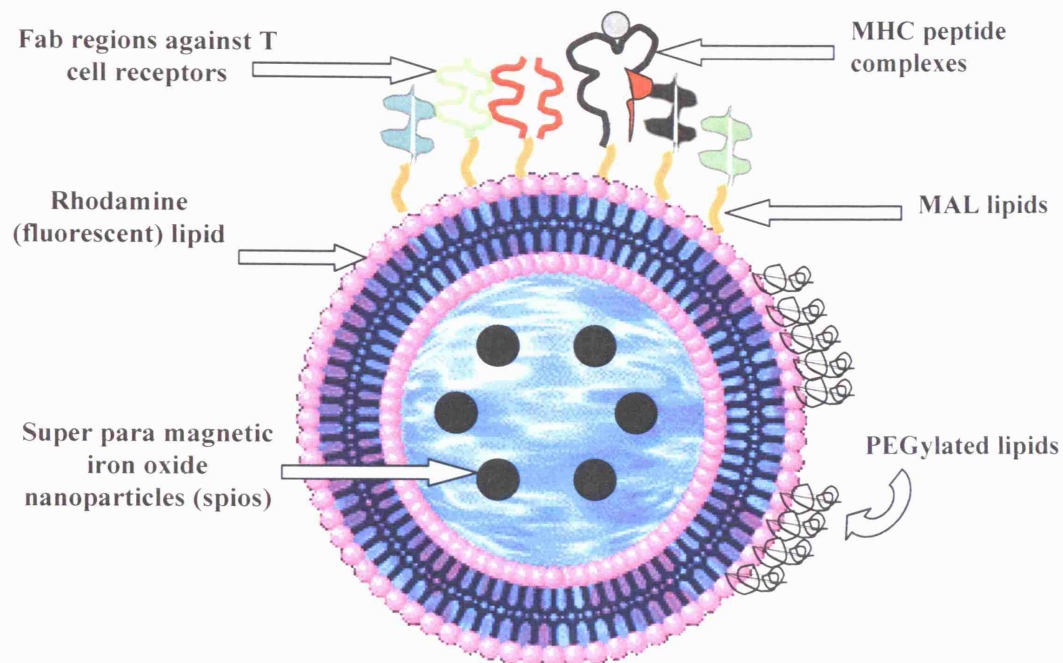


Figure 5.1.

ARTIFICIAL NANOTECHNOLOGY AND SUPER PARA MAGNETIC APC: Coated immuno magneto liposomes were generated to create an artificial APC for either adoptive and active immunotherapy. The scheme shows a double traceable APC generated by the incorporation of a fluorescent (rhodamine) lipid in the constructs plus the loading of magnetic resonance imaging (MRI) traceable spios in the inner compartment. On the surface, via MAL (maleimide) lipids, MHC peptide complexes, antibodies and or Fab regions against T cell receptors were incorporated to provide immunological functionality (anti LFA-1 as an adhesion molecule, anti CD28 and anti CD27 as early activation signals, anti CD40L to attract CD4 help and also as a survival signal and anti 4-1BB as late activation signal). PEGylated lipids ensure long circulating *in-vivo* times by avoiding uptake by the reticulo endothelial system.

(Yewdell and Hill 2002; Mocarski 2004). We have shown in chapter 4 that these artificial APCs only expand antigen specific T cells functioning either directly as micro APCs or semi-directly via natural APCs in a similar fashion to exosomes. Therefore, in order to assess the feasibility of active immunotherapy as a potential future use for this artificial APC, *in vivo* studies were performed. Pursuing active immunotherapy as one of the future applications for the system made us choose an appropriate carrier. Liposomes were chosen as the basic APC structure because they have been used *in vivo* in humans clinical trials previously (1995; James 1995) and because of the knowledge that they traffic in the human body safely. Studies in animal models have also shown their benign nature (Vingerhoeds, Storm et al. 1994; Park, Hong et al. 1997). They offer “off the shelf” availability and are generated in less than 12 hrs and as full aAPCs in less than 48 hrs as described in chapter 3. According to nanotechnology requirements, they were made traceable *in vivo* using super para magnetic iron oxide nano particles or spios, which can be followed by magnetic resonance imaging (MRI) and can be magnetically targeted to specific body areas such as the tumour site or particular lymph node regions with the use of external magnets as explained later. Immunoliposomes or targeted liposomes carry several different proteins on their surfaces and their lipid composition can vary according to their particular requirement. Therefore their *in vivo* kinetics depend on the type of lipids in the constructs such as PEGylated lipids, which assist in their avoidance of recognition and clearance by the reticulo endothelial system, the ratio of each lipid, their physical size, their route of administration and finally, what they are carrying on their surface. Therefore, under animal house regulations, mice studies were performed to determine the liposomes *in vivo* behaviour through the use of fluorescence microscopy and magnetic resonance imaging as described subsequently.

5.2 ASSESSMENT OF *IN-VIVO* aAPC PRESENCE BY FLUORESCENT MICROSCOPY

Using mice as an experimental model allows the translation of work into an *in vivo* setting as a prerequisite for eventual clinical studies. Balb/c mice were chosen because they are albino mice (figure 5.2), therefore are easier to inject since their tail veins are more visible. Balb/c mice are of low cost to maintain, have good breeding performance with large litters and short gestation periods. Following the animal house regulations (Animal Scientific Procedures Act 1986), the mice were intra-venously (i.v.) injected using the tail veins after a short period of vasodilation in 37°C chambers. An anaesthetic cream was applied topically on the site of injection and 200 µl of fluorescent un-coated liposomes were initially injected in duplicate. The mice were sacrificed by asphyxiation at different time points: 2hr, 24hr, 48hr and 72hr post injection. Blood samples were taken at each time point as well as sections from liver, spleen, lymph nodes, kidneys, gut, heart, brain, skin and lungs. The samples were cryopreserved, fixed and sectioned for analysis under fluorescence microscopy. Blood samples taken after 2 and 24 hrs post injection showed large amounts of liposomes in the circulation (figure 5.3). Liposomes were distributed homogeneously throughout the mouse body. Organs with higher blood supply such as liver, heart and lungs showed higher amount of liposomes (figure 5.4 and 5.5). However all organs analysed showed liposomes to a certain degree within the tissue and in the blood vessels. Liposomes were eventually cleared from the circulation by day 2. However liposomes remained in tissues for up to 3 days. At day four, the spleen remained as the only organ showing the presence of small amounts of liposomes within it (figure 5.6).

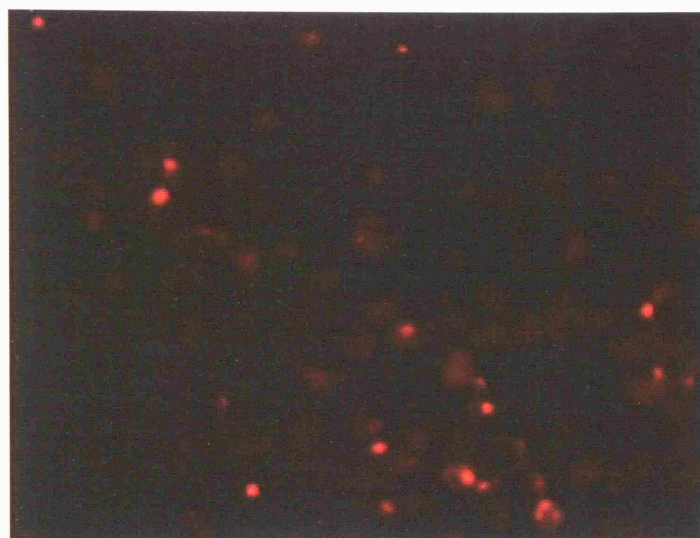
We then injected fluorescent-coated liposomes (aAPCs). Again duplicates were i.v. injected and sacrificed at the same time points, blood and organs were harvested, cryopreserved, sectioned and analysed by fluorescence microscopy. Figures 5.7 and 5.8 show the tissue distribution of coated liposomes. Less amount of coated liposomes were found in organs when compared to mice injected with un-coated liposomes. However coated liposomes were still detected in all tissues analysed. It



Figure 5.2.

***IN VIVO* MOUSE MODEL:** Balb/c albino mice were chosen as model for the *in vivo* studies. They were kept and handled under animal house regulations according to the Animal Scientific Procedures Act 1986.

A



B

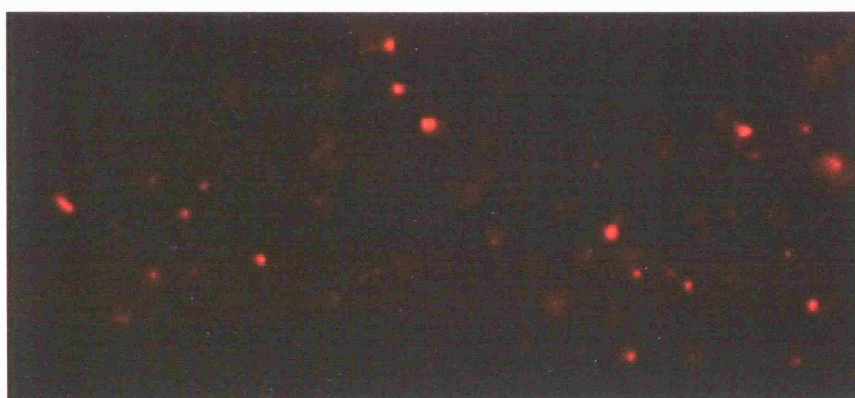


Figure 5.3.
MOUSE PERIPHERAL BLOOD *IN VIVO* PRESENCE:
Fluorescent uncoated liposomes in mouse peripheral blood following **A.**- 2 hrs and **B.**- 24 hrs post injection.

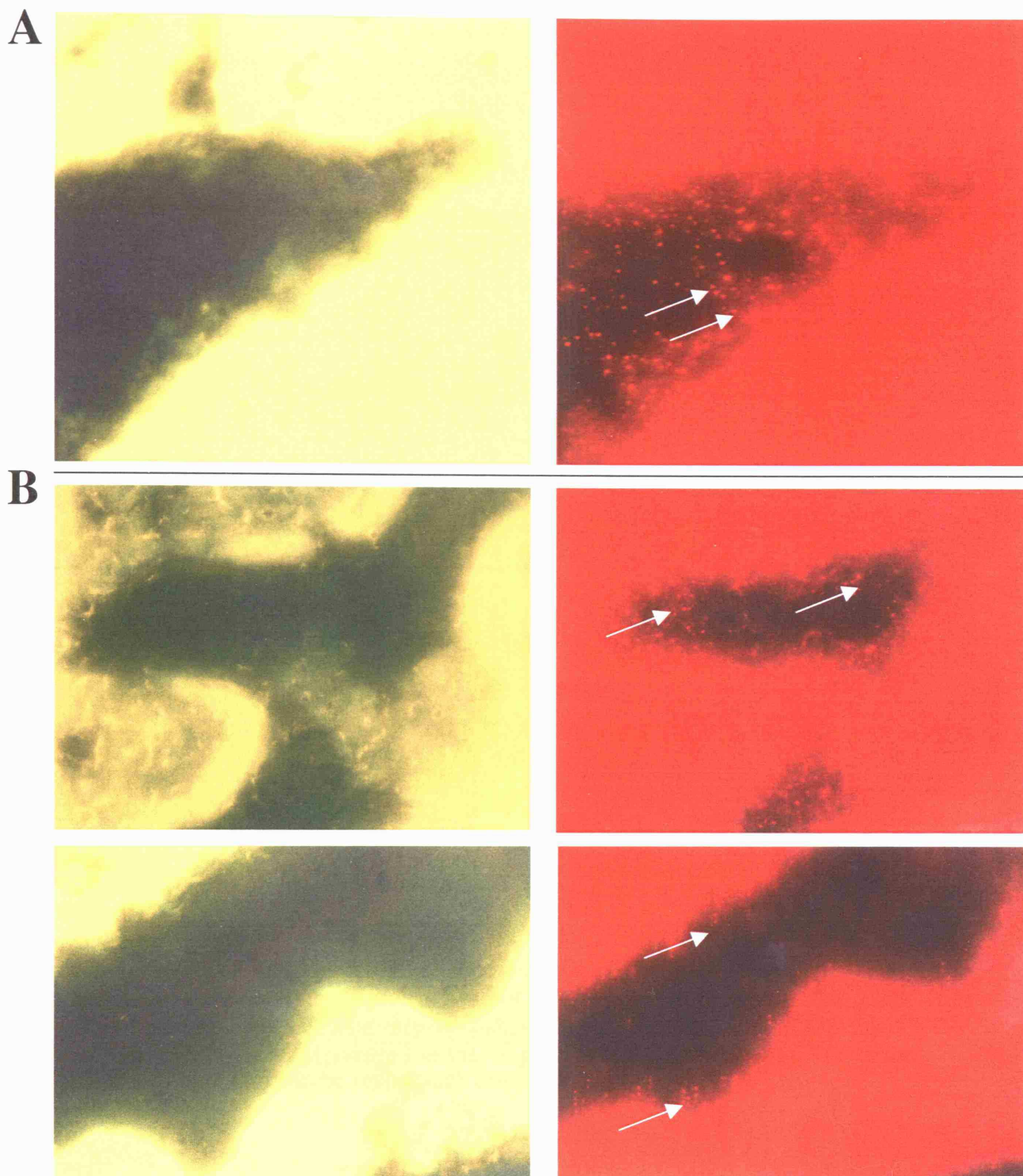


Figure 5.4.

MOUSE LIVER AND HEART BLOOD VESSEL *IN VIVO* PRESENCE: Fluorescent uncoated liposomes (white arrows) in blood vessels of **A.- Heart** and **B.- Liver** 24 hrs post injection. There is a reasonable level of auto-fluorescent by the tissues. Therefore an irrelevant fluorescent (green-yellow) picture from the exact same correspondent section is shown on the left hand side. However the liposomes can clearly be seen in the vessels as distinct objects on the right hand side pictures.

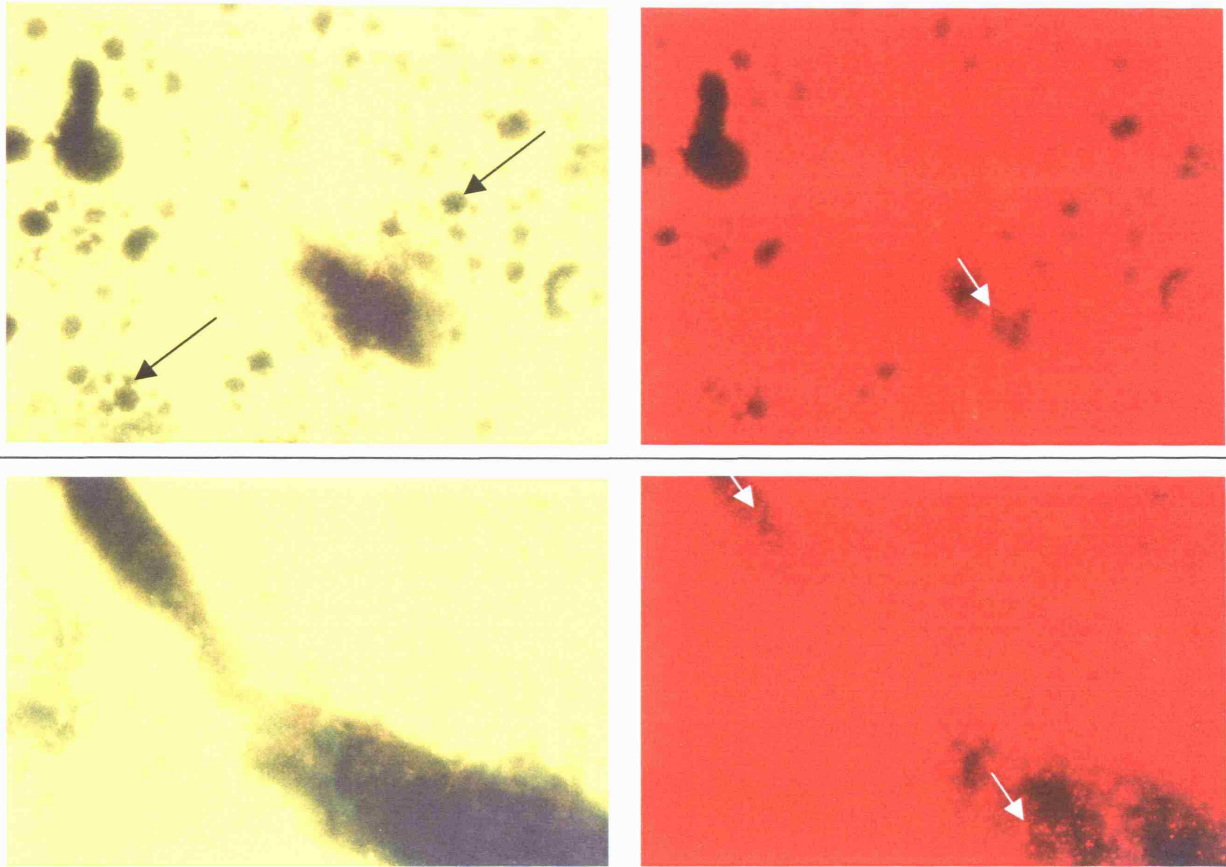


Figure 5.5.

MOUSE LUNG BLOOD VESSEL *IN VIVO* PRESENCE: Fluorescent uncoated liposomes (white arrows) in lung blood vessels 24 hrs post injection. Some organs as previously mentioned are auto-fluorescent. Therefore an irrelevant fluorescent (green-yellow) picture from the same section is shown on the left hand side to demonstrate the true presence of fluorescent liposomes in the lungs (right hand side picture). The structure of the alveoli compartment can be seen (black arrows).

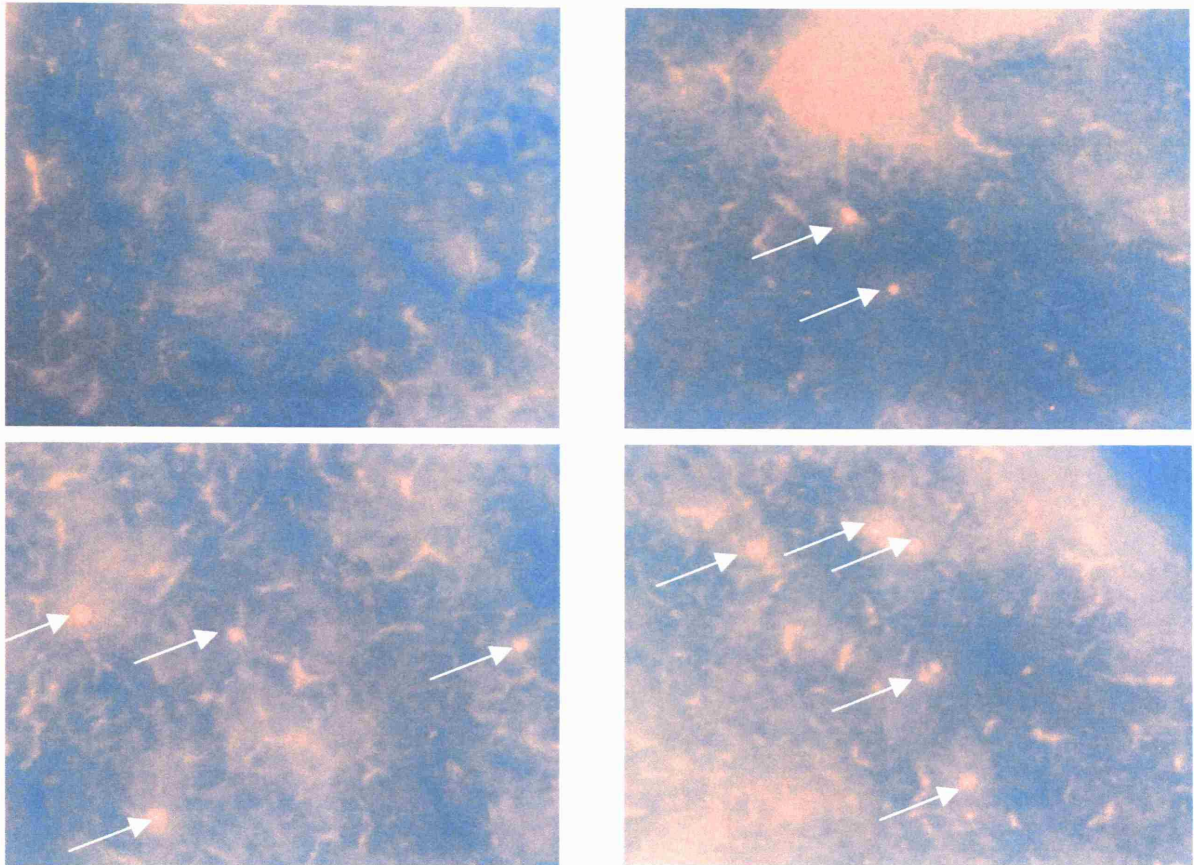


Figure 5.6.

MOUSE SPLEEN *IN VIVO* PRESENCE: **A.-** Section of the spleen of a control mouse. **B, C and D.-** Show different spleen section of a mouse injected with fluorescent uncoated liposomes (white arrows) 4 days post injection.

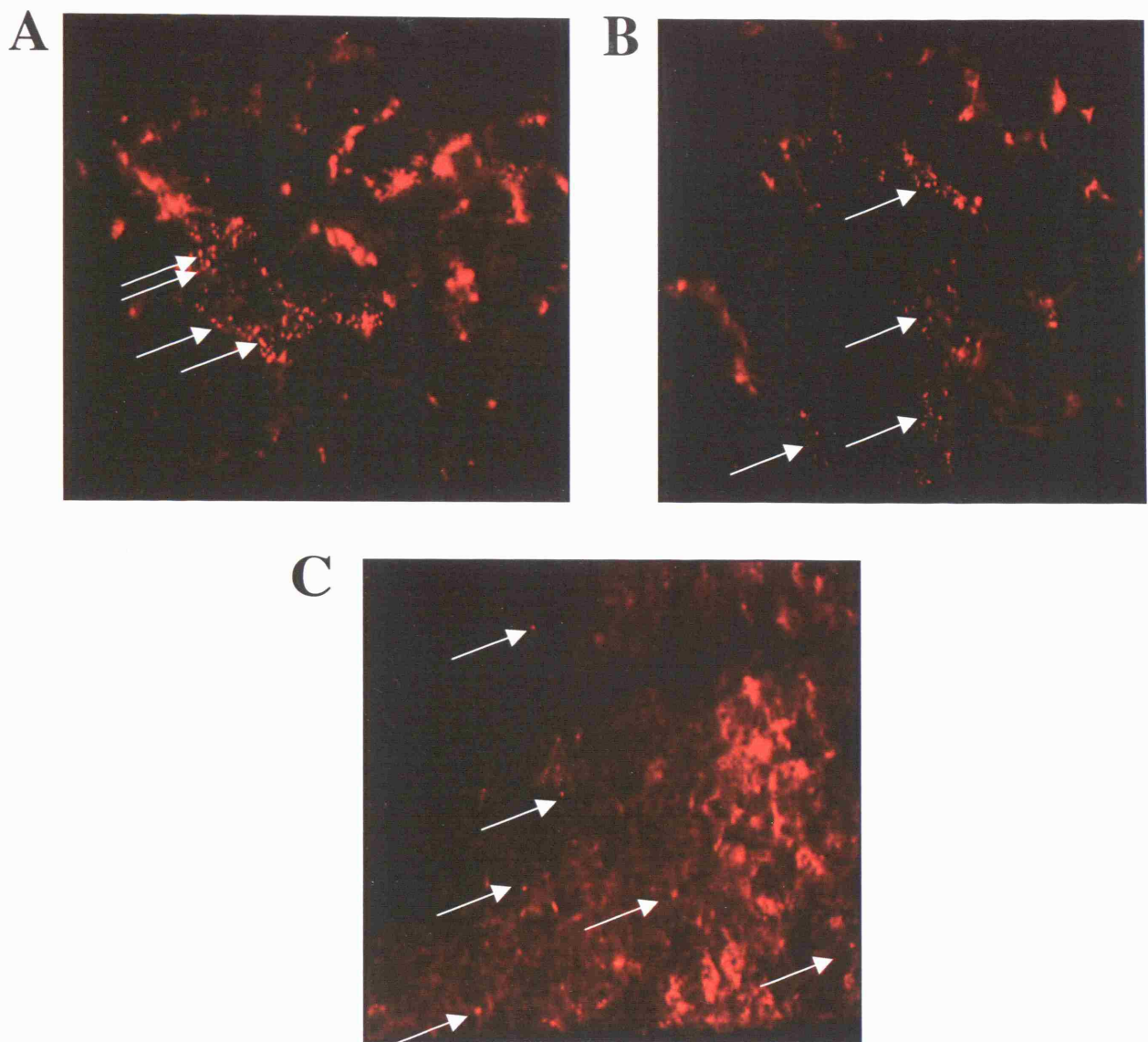


Figure 5.7.

aAPC *IN VIVO* PRESENCE: Coated fluorescent liposomes bearing murine mAbs against human receptors and human MHC peptide complexes were detected within the mouse tissues (white arrows) 24 hrs post injection. The fluorescent images above show a histological section from **A.- Lungs** **B.- Kidney** and **C.- Spleen**.

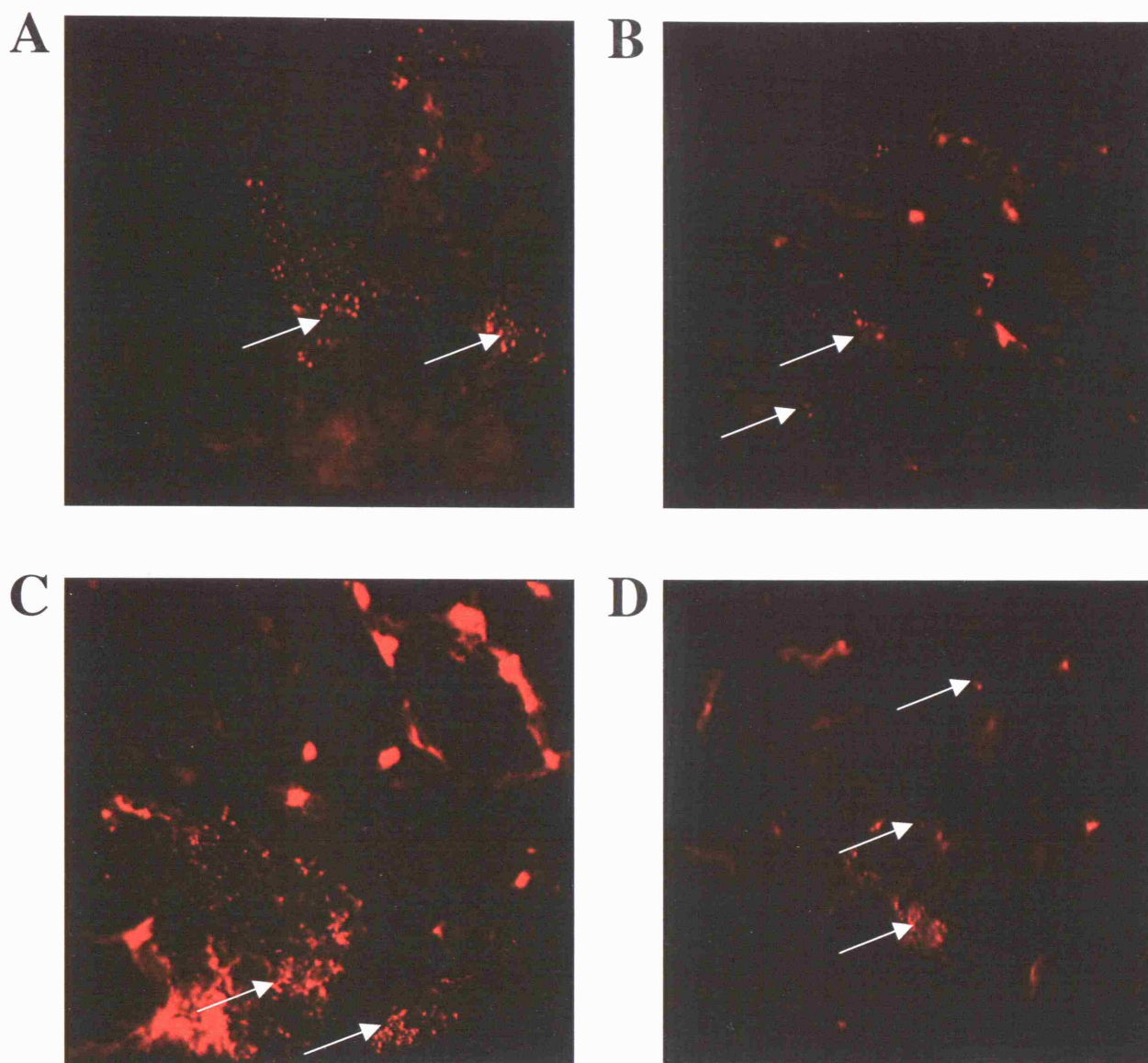


Figure 5.8.

aAPC *IN VIVO* PRESENCE: Coated fluorescent liposomes bearing murine mAbs against human receptors and human MHC peptide complexes were detected within the mouse tissues (white arrows) 24 hrs post injection. The fluorescence images above show a histological section from **A.- Liver** **B.- Heart** **C.- Gut** and **D.- Lymph node**.

is important to highlight that these coated liposomes injected in the mouse model were carrying molecules against human receptors (MHC peptide molecules and mAbs). This means that functionality of the liposomes as aAPCs could not be assessed in this system as explained subsequently. This model was used to assess flow dynamics despite the fact that the mAbs on the surface of the liposomes were to human receptors. Coated liposomes should have been protected from recognition to a certain extent by the PEGylated lipids from the liposomes, which helps avoid fast recognition by the reticulo endothelial system as explained in chapter 2. The use of Fab antibody regions rather than total monoclonal antibodies should also have a significant impact on the kinetics of the constructs as FC antibody regions are recognised by many cells and possess a lot of the immunogenicity of the molecules. Even so, the experiments performed using these more immunogenic constructs carrying whole antibodies showed distribution of coated liposomes in all tissues analysed. Concerning the MHC peptide constructs, these recombinant molecules are completely human and they could have been very immunogenic in a mouse model, which could affect significantly their kinetics and explain (together with the immunogenicity of the mAbs) why less overall amount of coated liposomes were found in different organs. However MHC peptide molecules recognition should have also had a certain level of protection by PEGylated lipids, which allowed the coated liposomes to be detected in all tissues analysed. Clearance of coated liposomes from the circulation took approximately 12 to 24hrs. By making fluorescent liposomes and taking in consideration that human proteins and whole mAbs were in the constructs, the aAPC *in vivo* presence was examined and was found to be similar to those of other immunoliposomes used *in vivo* in other studies (Maruyama 2002; Pastorino, Brignole et al. 2003). According to our results, coated liposomes in this model have an average half-life of 2 to 3 days, which should modify and increase with constructs coated with Fab regions and the MHC mouse counterpart molecules. Even with these parameters, it is expected that theoretically the aAPCs will have enough time to work as aAPCs *in vivo*. Experiments will be carried out on a T cell receptor (TCR) transgenic mouse model in the near future in collaboration with Prof. Hans Stauss at the Royal Free

Hospital, Immunology department. This TCR transgenic mouse model possesses T cells that recognise mouse MHC peptide molecules, which will be presented by coated liposomes. The mice will be tested to explore whether *in vivo* expansions can be accomplished following aAPC administration.

5.3 MRI *IN VIVO* aAPC STUDIES

In the previous experiments, fluorescence microscopy was able to detect coated fluorescent immunoliposomes as aAPCs in all tissues analysed. Therefore fluorescence microscopy could also detect the aAPCs in tumour biopsies and lymph node biopsies in order to determine whether activation of effector T cells takes place either in the circulation or *in situ* at the tumour site, and whether activation of naïve T cells takes place at the lymph nodes. That in itself would give invaluable information. However nanotechnology requires devices that can be detected not only *in situ* on fixed samples, but in real time or “live detection”. This is of paramount importance as live detection not only informs about *in vivo* interactions as they develop, but also allows the study of the nano device in inaccessible organs that are difficult or inconvenient to take biopsies from such as the brain and eyes. For safety reasons as well, *in vivo* follow up determines whether a system behaves properly or not. Therefore nanotechnology requires traceable *in vivo* devices in order to detect any predicted and unpredicted possible *in vivo* interactions.

Magnetic resonance imaging (MRI) is a fairly new technology. It started at the beginning of the 1980s and since then, it has revolutionised the way physicians and researchers diagnose and follow diseases. MRI gives exact details of all internal organs with great precision. Most importantly, there are no known dangers connected to MRI. The most important quality of MRI is that this Nobel Prize winning technology (2003) uses magnetic radio waves, which means that there is no exposure to X-rays or any other damaging form of radiation. These magnetic radio waves used by MRI scans affect the cells’ atoms. Our bodies mainly consist of water, and water importantly contains hydrogen atoms or protons. The excitation of the hydrogens’ protons is what creates the MRI images and these images can be

generated in two time constants modes: T1 and T2 (Callaghan 1991). The radio frequency excites the protons forcing them to spin around their axis giving them an angular orientation and producing magnetic fields (T2). As they recover and move back into place, they send out radio waves of their own, which are picked up by the scanner and converted into a picture (T1). Images in T1 are generated by the relaxation process due to the return of the high-excited protons to the low energy state, that generates an image in which a dark colour represents calcium (bones), whereas a bright or white colour represents lipids and soft tissues (McRobbie 2003). Tissues with the least amount of hydrogen such as bones turn out dark, whereas tissues rich in hydrogen such as fatty tissues like the brain look bright. Images in T2 are the exact opposite; dark means soft and white means bones.

As explained in detailed in chapters 2 and 3, traceable MRI immunoliposomes were constructed by entrapping super para magnetic iron oxide nanoparticles or spios (Khalafalla 1980) (which are detected by MRI scanners) inside the liposomes (figure 5.9). Firstly, concerning the safety of spios, this has been well established as they have been found in all live species from bacteria to fish, birds and humans (Kirschvink, Kobayashi-Kirschvink et al. 1992; Bazyliniski and Frankel 2004). Their production and functions are believed to be related to external magnetic orientation, and spios (in humans) are eventually converted into the “haem” groups of haemoglobin as part of their metabolism. Recently scientists have started to exploit them for scientific and medical purposes. Spios are not magnetic themselves. However they become magnetic when external magnets are applied to them. Due to their iron content, free spios preferentially target liver and bone marrow (Bulte, de Cuyper et al. 1999; Bulte and De Cuyper 2003). Therefore free spios have been injected *in vivo* as means to enhance magnetic resonance of these organs. In animal models, spios entrapped in liposomes, also called “magnetoliposomes” (Martina, Fortin et al. 2005), have been used to magnetically deliver chemotherapy to tumours. Liposomes as previously explained, target tumours more efficiently than the free drug due to the Enhanced Permeability and Retention tumour effect (EPR)(Fang, Sawa et al. 2003). Magnetoliposomes however, can be further concentrated at the tumour site using external magnets

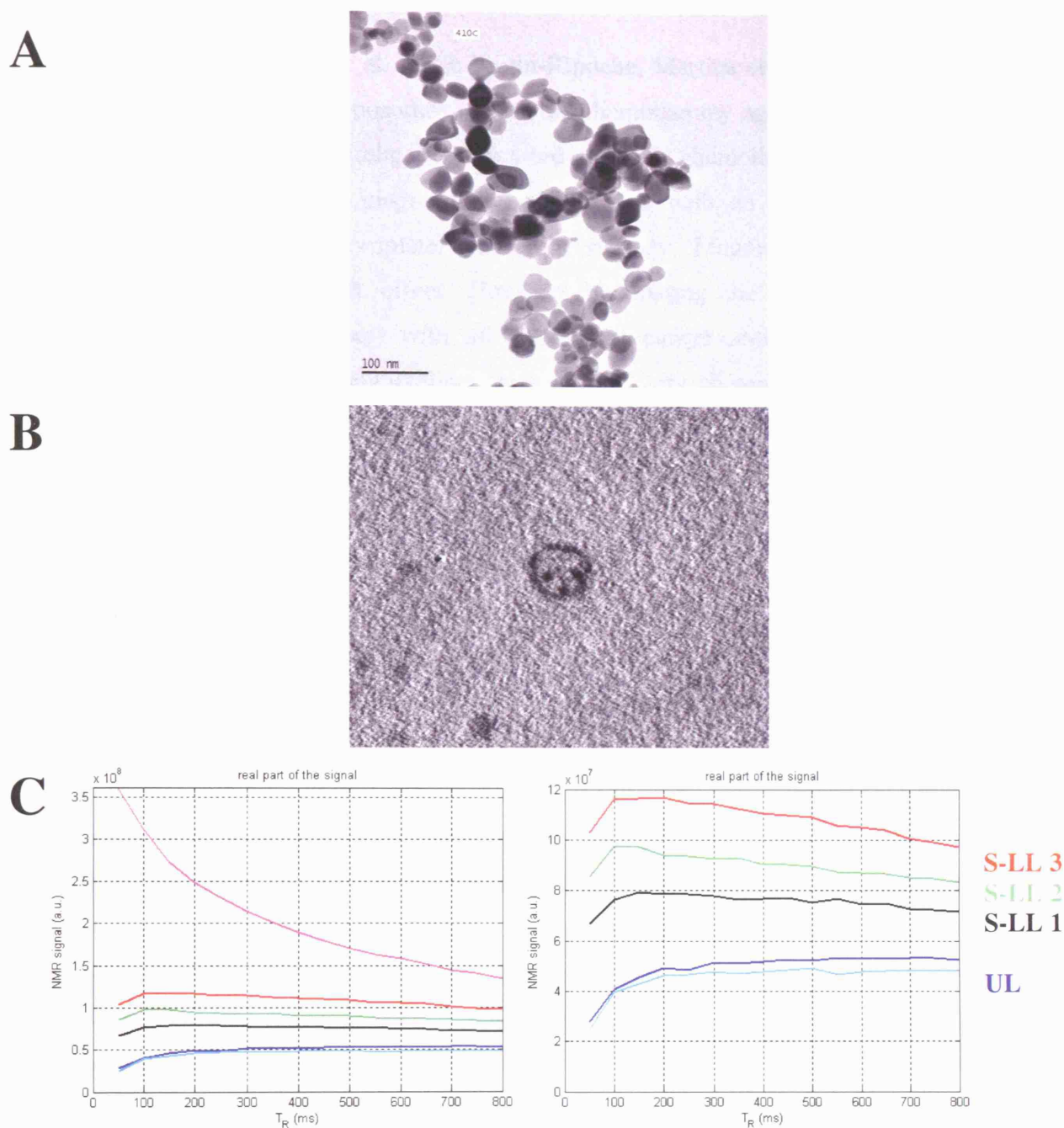
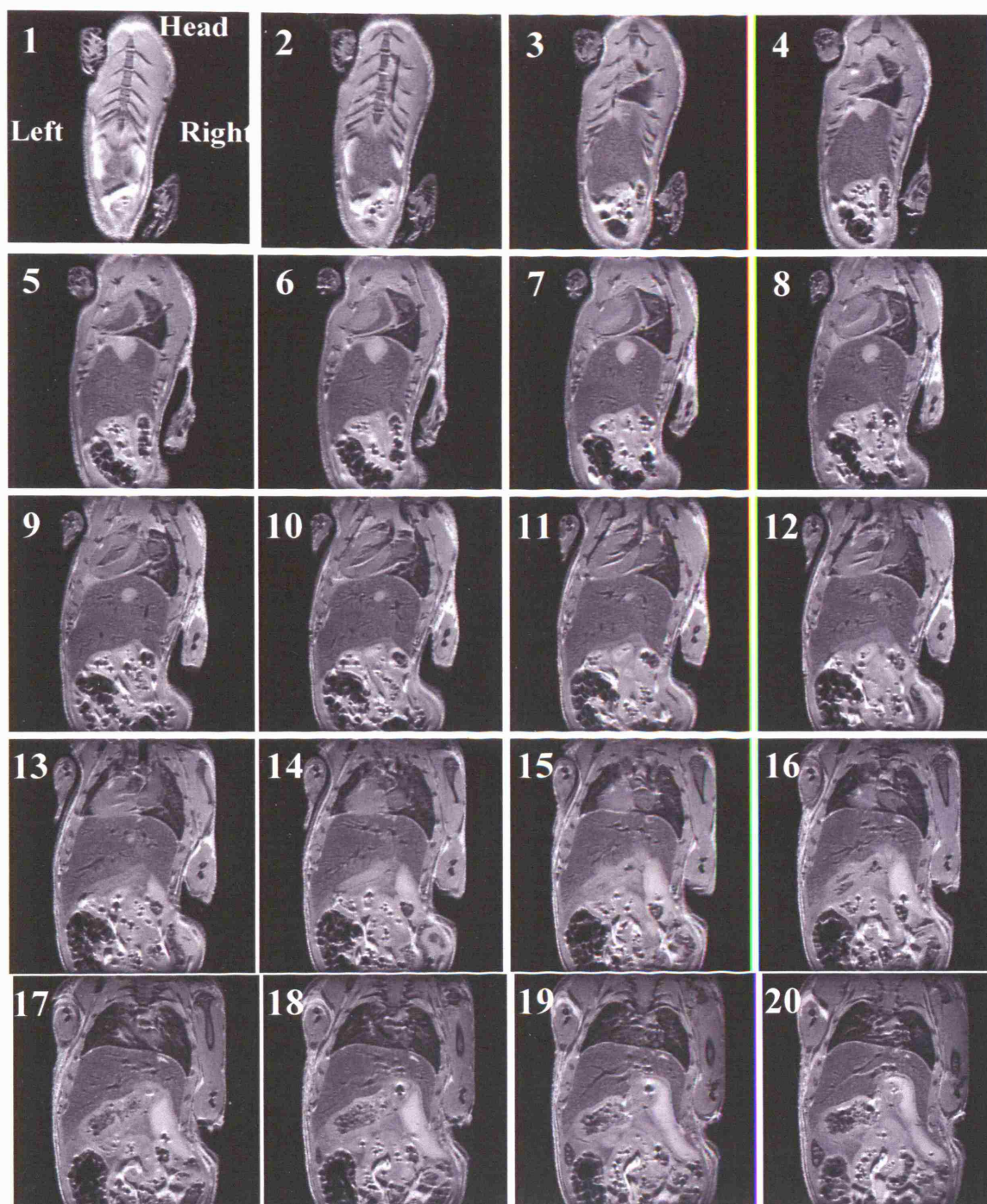
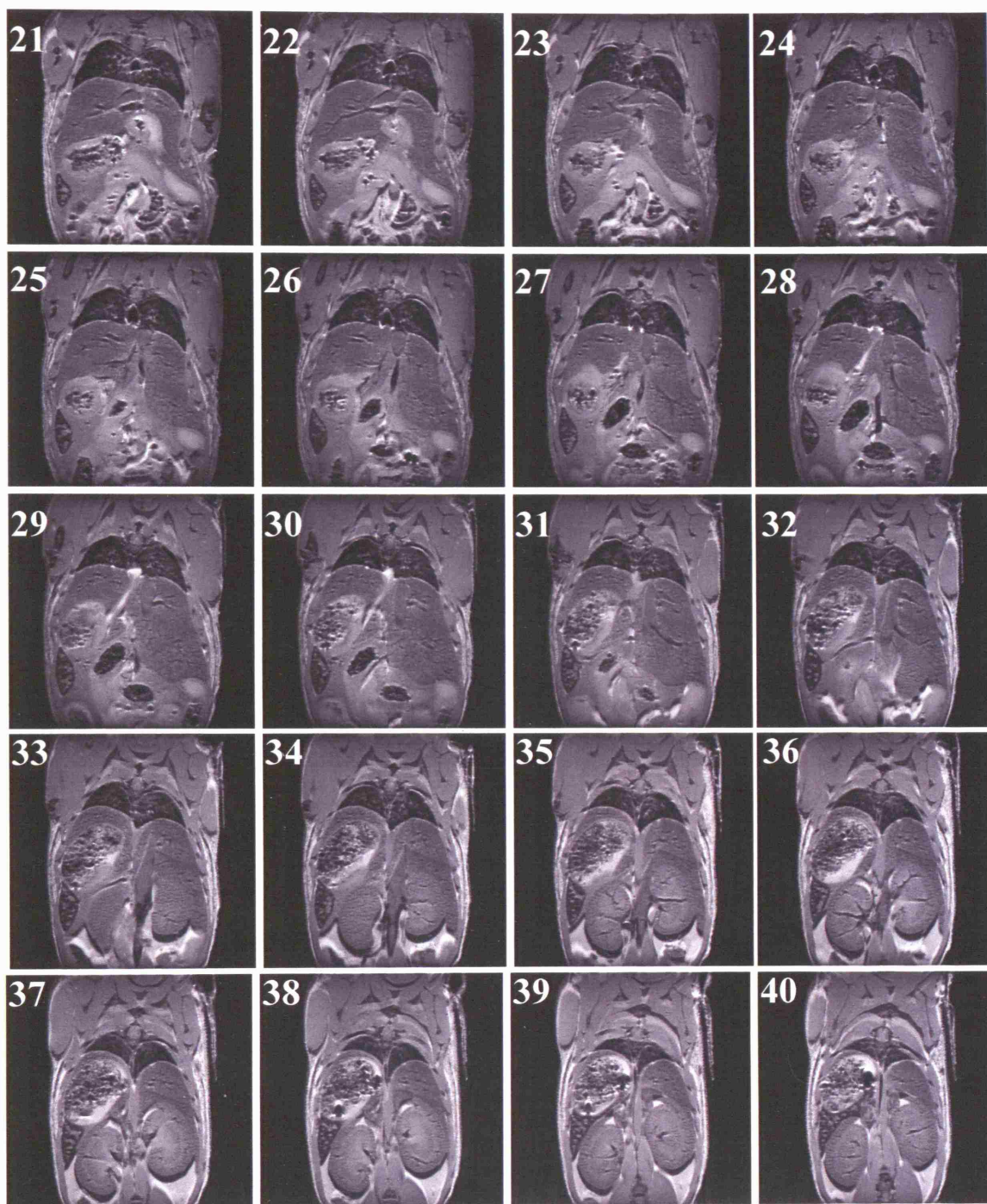


Figure 5.9.

MRI *IN VIVO* TRACEABLE aAPC: Super para magnetic iron oxide nano particles or spios (**A**) can be detected by magnetic resonance imaging (MRI) and can be concentrated to specific areas by using magnetic fields. In order to create an *in-vivo* traceable aAPC, liposomes were loaded with spios **B**. - Shows a liposome containing 3 spio particles viewed by electron microscopy. Following chromatographic separation of non entrapped spios, loaded liposomes were concentrated using magnetic columns and analysed on a mini MRI detector (**C**). The right hand side graphic is a zoom of the one on left hand side. Buffer and unloaded liposomes (UL) did not emit any MRI signal whereas spio-loaded liposomes (S-LL) in three increasing concentrations emitted a strong and powerful signal allowing the artificial APCs to be detected by MRI.

(Babincova, Altanero et al. 2000; Fortin-Ripoche, Martina et al. 2006). Once at the tumour site, magnetoliposomes release the chemotherapy agents accomplishing specific delivery and avoiding the unwanted systemic chemotherapy side effects. Other groups have used magnetoliposomes coated with an anti-HER2 (breast cancer) antibody as a completely different strategy. Magnetoliposomes target tumours due to the EPR effect. However by coating the magnetoliposomes (immuno-magnetoliposomes) with an anti breast cancer antibody, targeting is improved further. Such magnetoliposomes do not carry chemotherapeutic agents. Instead once these magnetoliposomes are concentrated at the tumour site, a magnetic field is applied, which produces hyperthermia locally as the iron from the spios inside the liposomes becomes excited and as a consequence the cells overheat (42.5 °C) and strong cytotoxicity is induced (Ito, Kuga et al. 2004). These are examples of how magnetoliposomes are being currently used. Our approach is to use immuno-magnetoliposomes to generate an artificial APC that firstly, can be magnetically targeted to particular places such as the tumour site or lymph node areas to accomplish T cell *in vivo* activation, and secondly, to generate an APC system that can be detected and followed in a “live” *in vivo* situation. For these reasons Balb/c mice were once again injected with liposomes either loaded or not with spios. Figure 5.10 shows a complete high definition MRI study from a control mouse, which shows all organs. Figure 5.11 shows a few MRI cuts from a mouse sacrificed an hour post injection with spio-loaded liposomes or magnetoliposomes. Once the injected mouse is inside the magnet, the magnetic field generated comes from all different angles in a circular manner. Therefore the magnetoliposomes do not move or follow any magnetic field during the study. They simply stay where they are at that moment. The intense red signals come from loaded liposomes and the MRI detection of loaded liposomes was indeed successful. Images from the portal system in the liver and the heart are shown. As proof of principle, the figures show that magnetoliposomes were indeed detected by MRI *in vivo*, which hopefully will open a vast range of scientific avenues for the use of this artificial antigen presenting cell system in the future of active immunotherapy and cancer nanotechnology.





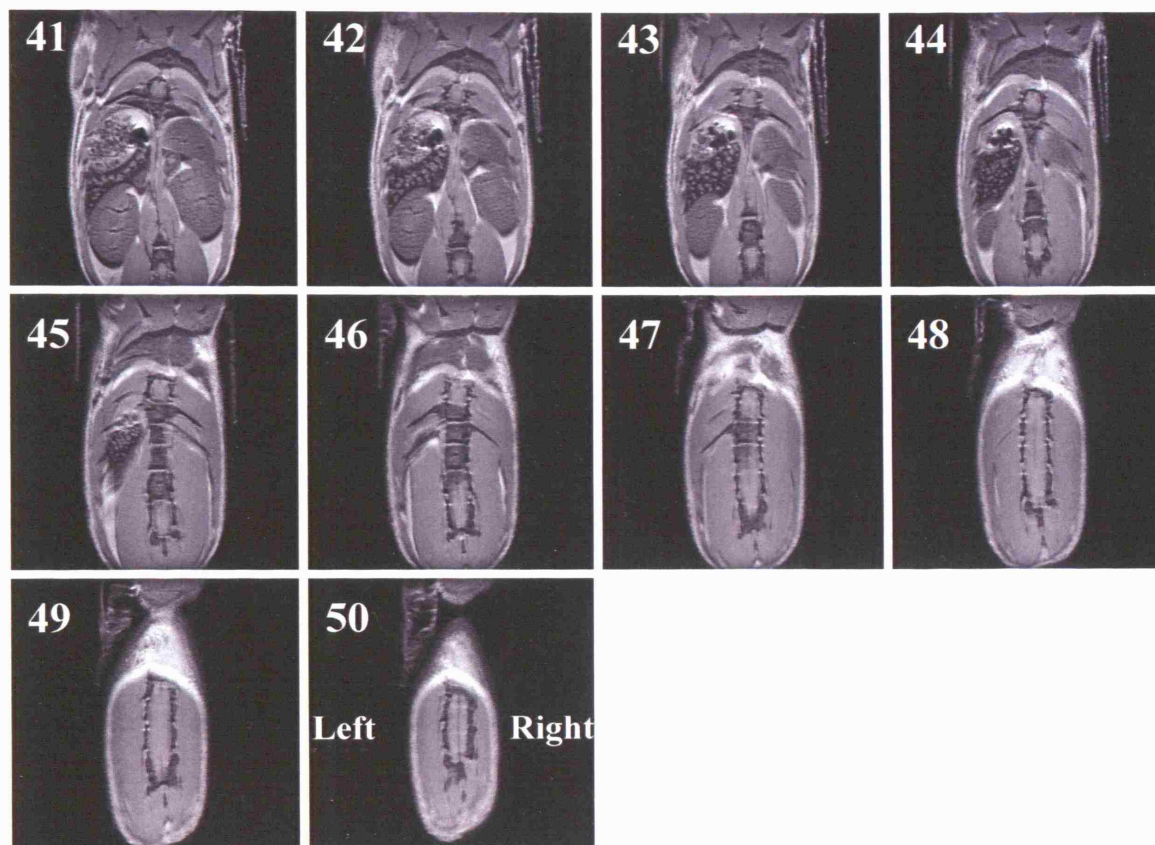


Figure 5.10.

HIGH RESOLUTION MRI (T1): Coronal MRI cuts from a mouse control. The mouse is seen from behind. The cuts however (1 to 50) progress from the front of the mouse to the back.

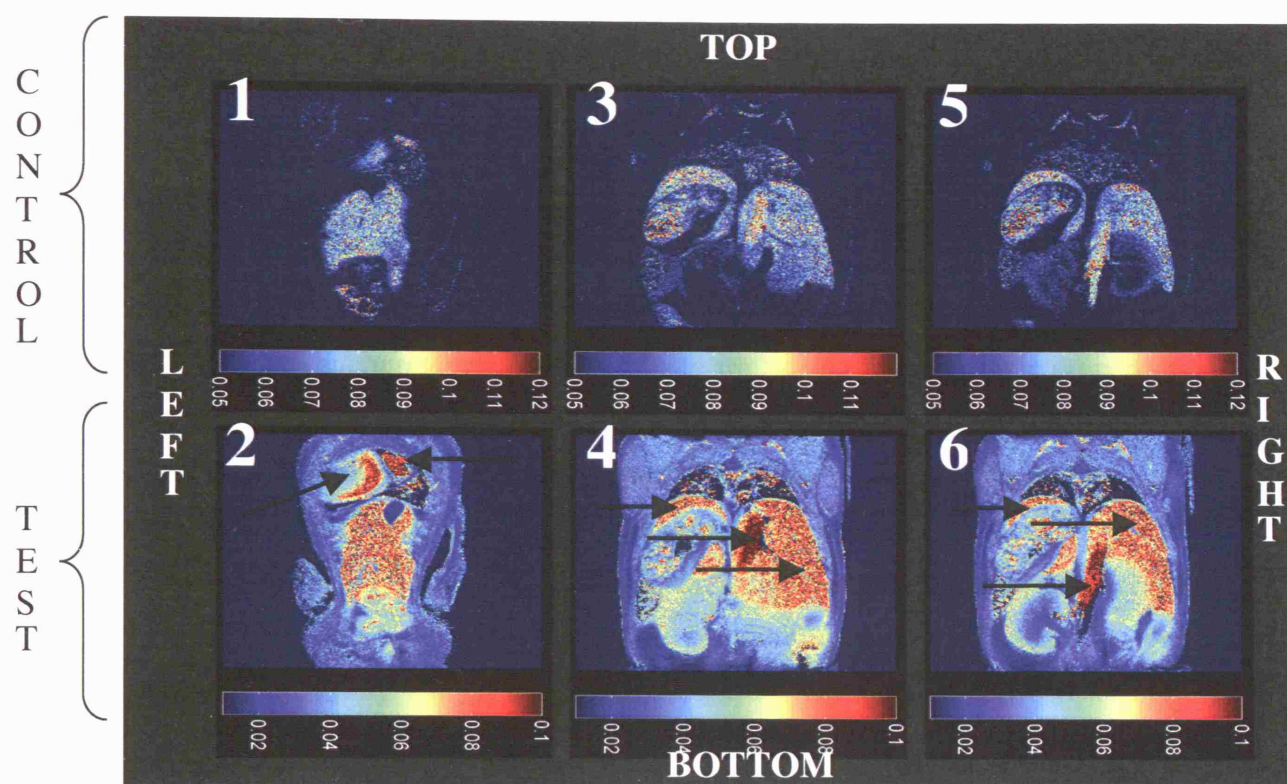


Figure 5.11.

aAPC MRI IN VIVO DETECTION: Figures shown above are colour enhanced MRI images. Liposomes loaded with spios (magnetoliposomes) were injected in a mouse (Test Mouse), which was sacrificed an hour post injection. The control and test mice are seen from behind in coronal cuts. **2.-** Shows the presence of magnetoliposomes (intense red) within the cardiac auricles and ventricles (arrows). No signal was detectable in the scan from the control mouse (1). Panels **4** and **6** show the presence of magnetoliposomes in the portal system, liver and spleen (arrows); panels **3** and **5** are the corresponding control scans.

Note: A high NMR relaxation rate, towards the red colours, corresponds to an NMR signal that disappears quickly, and occurs when loaded liposomes are present. A low NMR relaxation rate, towards the blue colours, corresponds to an NMR signal that remains measurable for a long time, and reveals the absence of loaded liposomes.

The MRI signal intensity in the test mouse has higher pixel intensity (≥ 0.05) in order to make the differences clearer.

In vivo T cell expansions will be studied in the near future in a TCR transgenic mouse model engineered to recognise mouse MHC/peptide complexes as explained previously.

5.4 CONCLUSIONS

These results show the feasibility for the use of the artificial antigen presenting cells for active immunotherapy since traceable *in vivo* aAPCs were successfully generated. Their flow dynamics behaved similarly to other liposomes used *in vivo*, which was also encouraging due to their safe trafficking and non-toxic properties. The aAPCs circulated through the mouse's body in an unrestricted fashion and were found in all tissues analysed from superficial tissues such as skin to protected organs such as the brain. They were found in small structures such as lymph nodes to high blood supported organs such as heart, lungs, liver and spleen, 2 to 3 days post injection; the aAPCs were detected by fluorescence microscopy. *In vivo* functionality of the system remains to be explored. However the *ex vivo* results are very encouraging (chapter 4). Therefore we have developed an artificial APC with enormous encouraging potential for active immunotherapy and cancer nanotechnology. The constructs are double labelled, fluorescent and super para magnetic liposomes are easy to produce since a small amount of fluorescent rhodamine labelled lipid is required to make the fluorescent constructs. Super para magnetic iron oxide nano particles or spios are easy to produce, non-expensive and unlimited amounts are generated at once reasonably rapidly. Regarding the MRI technology, even though expensive due to the scan-magnet size, offers a non-invasive tracking system which gives the greatest detail ever accomplished for live detection within live organisms. Hopefully this aAPC system will provide an improved targeted tool for active immunotherapy in the near future.

CHAPTER 6

CONCLUSIONS

6.1 SUMMARY AND GENERAL CONCLUSIONS

Data presented in this thesis describe in a detailed and comprehensive manner the development and construction of a novel artificial antigen presenting cell system projected for both *ex vivo* and *in vivo* clinical applications. Chapters 2 and 3 describe how the nano APC system was generated in less than 48 hr by successfully coating liposomes with well orientated and refolded MHC peptide complexes, plus mAbs and Fab regions as co-activatory signals. These two chapters also show the generation of fluorescent and MRI traceable liposomes and their stability over time. Liposomes themselves remain intact for at least 14 days. Once the aAPCs are created however, they remain viable and stable for at least 7 days. This artificial APC system is a non-cell based system, which is not affected by many of the tumour and pathogen immune evasion and suppressive strategies responsible for the disease itself or the malfunction of the patient's natural APCs. The artificial APCs enter the body as fully mature APCs that do not require further antigen processing, which embodies a significant advantage when compared to vulnerable cell based APC systems.

Chapter 4 describes how through use of the system, reproducible antigen specific T cell *ex vivo* expansions (from 55 to 200 fold) in a memory setting have been achieved. This level of expansion is superior to those accomplished by the use of dendritic cells, which is the standard current approach and other artificial APC systems currently tried at the Anthony Nolan Research Institute such as modified tetramers, magnetic beads and coated Daudi cells. Functionality of expanded T cells was addressed by looking at the correlation between tetramer staining, degranulation assays, phenotypic characterisation and cytokine production. The cells mainly expressed effector-memory and memory phenotype, which are both

important to provide short and long term clinical effect. It has been shown that as a mechanism of action, the artificial APCs bind and trigger activation and expansion directly on T cells in an antigen specific fashion and also in a semi-direct fashion via natural APCs functioning in a similar manner to exosomes. And finally, chapter 4 also describes that with respect to the naïve setting, it has been possible to achieve what appears to be an initial level of successful T cell priming against CMV without the need of adjuvants as other systems use.

Chapter 5 describes how using a mouse model, the system is traceable *ex vivo* and *in vivo* by fluorescent microscopy and magnetic resonance imaging as the aAPCs carry fluorescent labels and super para magnetic iron oxide nano particles. This had the final goal of showing the feasibility of the system as an active immunotherapeutic approach, since liposomes are super-para-magnetic, that also allows their magnetic targeting to particular sites of interest in the body by applying external magnetic attraction. No toxicity was seen *in vivo* and good kinetics were confirmed. Even though the aAPCs were carrying human molecules, they were found in all mouse organs screened: skin, brain, lymph nodes, heart, lungs, liver, gut, kidneys and spleen, which implies that the generation of an aAPC-mouse counterpart should improve further their flow dynamics.

In vivo T cell expansions will be studied following this PhD in the near future using a transgenic mouse model engineered to recognise mouse MHC/peptide complexes, which will be presented by coated liposomes.

Overall however, we have standardised the conditions for an efficient artificial APC system, which embodies a controllable and superior approach with enormous potential for cancer nanotechnology and T cell mediated immunotherapy.

Liposomes for class II activation must be eventually produced in order to deliver CD4 help and killing. Tumour aAPC models and aAPCs for negative vaccination are also in the scope of this strategy as explained in the following section.

Immuno-magneto-liposomes represent a solid immunotherapeutic platform based on nanotechnology to deliver new superior treatment and monitoring strategies to patients.

6.2 FUTURE PERSPECTIVES

6.2.1 Liposomes Interior

Liposomes started as therapeutic agents for their capacity to carry substances in their liquid interiors. The options are unlimited: they can carry chemotherapy, DNA, RNA, peptides, cytokines, danger signals, adjuvants, antibodies, etc. Loading or entrapping cytokines for instance could deliver signals for either up and down immuno modulation to enhance immune responses generated by the liposome binding.

6.2.2 Tumour Antigen Liposomes

The aAPCs were generated to mount immune responses towards CMV antigens as proof of principle since the Anthony Nolan Research Institute is part of a bone marrow transplantation centre and therefore has considerable experience in this field. The CMV immunodominant peptides are well known and the kinetics of a normal response are also known. However liposomes coated with MHC/tumour peptides will be explored following this PhD in order to mount significantly relevant T cell responses that could deliver important clinical effects to cancer patients.

6.2.3 Class II aAPCs

It has been previously outlined the importance of CD4⁺ T cell help in the form of cytokines and cross-talk with natural and artificial APCs in the immunological synapse formation and for complete CD8⁺ T cell activation. Certainly CD4 mediated killing has been shown to be as important as CD8⁺ T cell mediated killing in some tumour models. Therefore liposomes carrying MHC class II constructs should also be constructed in order to expand both branches of the adaptive immune response and deliver a more complete immune response. Class II constructs are not

as easily produced as class I constructs. Protocols for their in-house production have been initiated recently and will be hopefully available in the near future. Class II liposomes will be then constructed and explored as a critical and complementary strategy to the class I counterpart already developed.

6.2.4 aAPCs For Negative Vaccination

Eventually this aAPC system can be also used in a specific way to inactivate or delete regulatory T cells, alloreactive or autoimmune T cell clones from the human body. In this case the liposomes would be coated exclusively with specific MHC/peptide complex or with a combination of MHC/peptide complexes specific for the target T cell clone plus Fab regions targeting inactivating or inhibitory molecules to induce anergy and/or programmed cell death such as CTLA-4, BTLA-1 or Notch. This particular system is beyond the scope of the activation model described here. However it remains a desired component for the future of this project and is currently being developed by other students as a continuation of this work.

APPENDIX

APPENDIX 1

Publications And Presentations

a) Publications

H. De La Peña, J.A.Madrigal, M.Bencsik, Gareth W.V.Cave, A.Selman, Rees RC, P.J. Travers, I.A. Dodi. Artificial Super Para Magnetic Nano APC For Active And Adoptive Immunotherapy. (Waiting submission).

H. De La Peña, J.A.Madrigal. Cell-Based Cancer Immunotherapy. Gaceta Mexicana de Oncologia Vol. 5, Num 3, may-june 2005.

b) Published Abstracts

H. De La Peña, J.A.Madrigal, M.Bencsik, Gareth W.V.Cave, Rees RC, P.J. Travers, I.A. Dodi. Artificial APC System For T Cell Adoptive And Active Immunotherapy. Blood (American Society Haematology ASH abstract) 2006.

H. De La Peña, J.A.Madrigal, M.Bencsik, Gareth W.V.Cave, A.Selman, Rees RC, P.J. Travers, I.A. Dodi. Immuno Magneto Liposomes As Artificial APCs For Active And Adoptive Immunotherapy. Progress In Drug And Vaccine Delivery International Liposome Society, ILS Abstract 2006.

H. De La Peña, J.A.Madrigal, M.Bencsik, Gareth W.V.Cave, Rees RC, P.J. Travers, I.A. Dodi. Hemato Nanotechnology: Artificial APC System For T Cell

Adoptive And Active Immunotherapy. Blood (American Society Haematology ASH abstract) 2005.

H. De La Peña, J.A.Madrigal, M.Bencsik, Gareth W.V.Cave, Rees RC, P.J. Travers, I.A. Dodi. Immuno Nanotechnology: Artificial APC System For T Cell Adoptive And Active Immunotherapy. Progress In Vaccination Against Cancer, PIVAC Abstract 2005.

H. De La Peña, J.A.Madrigal, M.Bencsik, Gareth W.V.Cave, Rees RC, P.J. Travers, I.A. Dodi. Immuno Magnetic Liposomes for Cancer Nanotechnology and Immunotherapy
In-Vivo. Progress In Drug And Vaccine Delivery International Liposome Society, ILS Abstract 2005.

H. De La Peña, S. Rusakiewicz, J.A.Madrigal, P.J.Travers, I.A.Dodi. Novel Artificial APC For *Ex-Vivo* And *In-Vivo* T Cell Priming And Expansion. Eur. J. Immunogenetics British Society of Histocompatibility and Immuogenetics, BSHI Abstract 2004.

c) Oral Presentation

Hugo De La Peña. Immuno Magneto Liposomes As Artificial APCs For Active And Adoptive Immunotherapy. Progress In Drug And Vaccine Delivery International Liposome Society ILS Meeting, London, UK, 2006.

Hugo De La Peña. Artificial Super Para Magnetic Nano APC For Active And Adoptive Immunotherapy. Royal Free Hospital, Medical School, UCL, London, UK, 2006.

Hugo De La Peña. Artificial Nanotechnology And Super Para Magnetic APC For Active And Adoptive Immunotherapy. Institute of Medical Immunology

Universitätsmedizin Charité, Berlin, 2006.

Hugo De la Peña. Artificial Nanotechnology And Super Para Magnetic APC For Active and Adoptive Immunotherapy. Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, 2006.

Hugo De La Peña. Immuno Nanotechnology: Novel Artificial APC For Adoptive And Active Immunotherapy. Progress In Vaccination Against Cancer (PIVAC) Meeting, Pasteur Institute, Athens Greece 2005.

Hugo De La Peña. Immuno Nanotechnology: Novel Artificial APC For Adoptive And Active Immunotherapy. Medical School, UAC, Tor, Mexico, 2005.

Hugo De La Peña. Novel Artificial APC For Ex-Vivo And In-Vivo T Cell Priming And Expansion. Progress In Vaccination Against Cancer (PIVAC) Meeting, Black Forrest Germany 2004.

Hugo De La Peña. Novel Artificial APC For Ex-Vivo And In-Vivo T Cell Priming And Expansion. British Society For Immunogenetics And Histocompatibility (BSHI) Meeting, Dublin, Ireland 2004.

Hugo De La Peña. Immune Gene Therapy: Artificial Cells, Magisterial Conference, Diagnostica Labs, Tor, Mexico, 2003.

Hugo De La Peña. Novel Artificial APC For Ex-Vivo And In-Vivo T Cell Priming And Expansion. Bone Marrow Transplant Meeting. Royal Free Hospital, UCL, London UK, 2003.

Hugo De La Peña. Immunomodulation And Immune Gene Therapy: aAPCs / T - Bodies. General Hospital, Sal, Mexico, 2002.

Hugo De La Peña. Immunomodulation And Immune Gene Therapy: aAPCs / T - Bodies. Medical School, UAC, Tor, Mexico, 2002.

Hugo De La Peña. Immunomodulation And Immune Gene Therapy. Bone Marrow Transplant Meeting. Royal Free Hospital, UCL, London, UK, 2002.

APPENDIX 2

Awards

Young Scientist Award. Prize given on behalf of The British Society For Histocompatibility And Immunogenetics, Dublin, Ireland 2004.

"Novel Artificial APC For Ex-Vivo And In-Vivo T Cell Priming And Expansion"

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